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Chemoreceptivity of *Drosophila melanogaster*

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(Received 16 August 1944)

The occurrence of mutants of *Drosophila melanogaster* distinguished by the absence or structural modification of the antennae provides a means of assessing the role of the antennae with respect to the reception of various classes of stimuli.

Antennaeless (A_0) phenotypes of *antennaeless* stock fail to respond to those chemical stimuli which lead the fly to its food. Their temperature reactions are normal, and their humidity responses are opposite to those of somatically wild-type flies of the same stock or of wild-type controls.

Aristapedia (as^a), which have leg-like antennae equipped with surface pegs and cones of supposed sensory function present in the normal antenna but absent in the normal leg, respond to chemical stimuli and humidity differences.

As compared with that of normal flies, the olfactory response of *aristapedia* (as^a) is somewhat less intense, the humidity reaction being somewhat stronger. These mutants do not give the characteristic responses evoked by thermal stimuli both in normal flies and antennaeless phenotypes.

The outstanding histological differences between the structure of the antenna of *aristapedia* and that of wild-type flies is the absence of the pit organ.

It thus seems that the pit organ is not essential to the olfactory response and plays no essential part in the humidity response.

Since *antennaeless* (A_0) responds normally to thermal stimuli, none of the putative sense organs of the antennae are essential to the recognition of temperature differences, and since *aristapedia* (as^a) responds more weakly to chemical stimuli than do normal flies, the pit organs may well be long-distance chemoreceptors.

What is more certain is that either the peg-like organs or the cones on the surface of the distal joint of the antennae or both are chemoreceptors. The same remark is equally applicable to the perception of humidity differences. Experiments here recorded do not justify the identification of the function of one or other type of sensilla with one or the other type of receptivity.

While it is unjustifiable to exclude the possibility that short-distance chemical stimuli play a part in the attraction of flies of opposite sex, it appears that the main role of chemoreceptivity in relation to the mating behaviour of *D. melanogaster* is to ensure the aggregation of flies of both sexes in situations where food is available and sexual congress can be evoked by other forms of stimulation.

1. INTRODUCTION

Investigations on the behaviour of mutants of *Drosophila melanogaster* have a twofold interest. On the one hand, more intimate knowledge of the reproductive habits of a species about whose genetic architecture we now know so much is a necessary prerequisite to the study of the role of assortative mating in the evolution of the group to which it belongs. On the other hand, such knowledge gives us an opportunity for taking advantage of structural modifications which may throw light on the function of putative sense organs in a group of insects too small for

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adequate study by electroneurological techniques; and it provides a method of investigating them without recourse to operative procedures beset by difficulties of several kinds. This advantage calls for little comment. Various authors, notably McIndoo (1914), have sufficiently stressed the uncertainties to which interpretation of experiments involving operative interference with this end in view are subject.

Among types of receptivity which claim priority in connexion with the study of mating behaviour, *photo-* and *chemoreceptivity* are pre-eminent. Present knowledge of chemoreceptivity among insects as a whole is meagre. On purely histological grounds, various authors (in particular, e.g. Smith 1919) have advanced the possibility that it may be largely localized in the antennae of some species. On the other hand, McIndoo (1914) has gone so far as to assert, on insufficient evidence as it seems to us, that this is never true. Such suppositions carry little conviction in the absence of *experimental* proof that structures of the same type located in the antennae or elsewhere have or have not the function ascribed to them; and conclusive proof of this sort is still lacking. Relevant information is available in a review by Marshall (1935), and a full summary of the position has been given by Wigglesworth (1939). The discovery by Gordon in 1938 of a new mutant of *D. melanogaster* characterized by absence of antennae therefore offered a unique opportunity for reopening the issue. Accordingly, experiments on the chemoreceptivity of this mutant began in the laboratory of the senior author in 1938. The outbreak of war made it impossible to develop a programme of work intended to embrace a study of the behaviour of mutants of *Drosophila* directed to both the objectives previously stated.

For investigation of the sensory function of the antennae of Diptera, or at least of *Drosophila*, the mutant *antennaless* has a particular advantage. For details the reader may consult the publication of Gordon & Sang (1941). In brief, the manifest somatic effect of the presence of the gene in duplicate may be bilateral, unilateral or *no* suppression of the antennae. That is to say, homozygous *antennaless* flies may have no antennae, one antenna or two antennae. We shall refer to such individuals by the symbols A_0 , A_1 and A_2 respectively. In the laboratory of the senior author of this communication, Gordon & Sang (1941) have explored some of those characteristics of the environment more or less propitious to exhibition of the gene. Begg & Sang (1944) have investigated others. It is now possible to produce at will, from inbred stocks exposed during larval life to different culture conditions, a higher or lower proportion of genetically identical individuals of the three types specified. Conclusions drawn from a comparison of their behaviour are therefore free from the objection that behaviour differences are ascribable to genes other than the gene *antennaless* itself.

In the normal cultures generally employed for genetic work on *Drosophila* mutants, *antennaless* is subject to an emergence effect. That is to say, the proportion of the three phenotypes, A_0 , A_1 and A_2 , varies during the life of a culture. It is unnecessary to take elaborate precautions to ensure high or low exhibition if the end in view is merely to maintain an adequate supply of any one of them for

experimental studies of the type described below. The homozygous stocks used were from those which Gordon & Sang had so maintained for over 100 generations. For certain purposes we have also used stocks homozygous both for the gene *antennaeless* and for the gene *vestigial*, inbred for at least fifty generations as such. All control stocks of other mutants were also highly inbred. The culture medium was a live yeast and agar-oatmeal-malt-extract gel.

Aristapedia, another mutant of *D. melanogaster*, is also important in connexion with the sensory function of the antennae. The outstanding characteristic of homozygous *aristapedia* (*ss^a*) stocks is replacement of the normal antenna by a leg-like appendage. In spite of this gross structural anomaly, microscopic examination of the modified antenna of the mutant reveals the presence of peg-like organs and of other structures of supposedly receptive function present in the normal antenna but not in the normal leg. We have therefore examined the responses of *aristapedia* as well as of *antennaeless* by the several procedures enumerated below.

2. METHODS

The main objective of the experiments we here record was the sensory function of the antennae with special reference to chemoreceptivity. At the same time, we have included in the scope of our inquiry experiments designed to elicit the extent to which receptors involved in the reactions to humidity and temperature may also be localized at the same site. A sufficient reason for undertaking such experiments is that no conclusion about the association of chemoreceptivity with particular micro-organs found in the antennae alone are necessarily legitimate, unless we have explored how far the sensory functions of the antennae are unique.

(a) *Chemoreceptivity*. To explore the extent of chemoreceptivity associated with the degree of exhibition of the gene *antennaeless*, two methods proved to yield consistent and conclusive results. We shall refer to these as the *trapping* and *two-way chamber* methods. The trapping method is essentially similar to that devised by Barrows (1907). It is well known that either yeast cultures or solutions containing acetic acid, ethyl alcohol, and various other organic compounds—in particular esters—exert an attraction for *D. melanogaster*. A mixture, here denoted *M*, containing bakers' yeast, ethyl alcohol, acetic acid and traces of other organic compounds, attracts the flies more strongly than any single constituent thereof. Its precise composition is as follows:

acetic acid	1 %
ethyl alcohol	16 %
methyl acetate	0.001 %
ethyl acetate	0.001 %
acetaldehyde	0.001 %
butyric acid	0.001 %
distilled water	to 100 %
bakers' yeast	3 g./25 ml.

The *trapping method* involves release of large numbers of flies of various phenotypes in an experimental chamber containing bottles with a capillary orifice (figure 1). For adequate controls, it is necessary to offer the flies a choice between equal numbers of bottles containing water (*W*) or the bait (*M*). What we here call the *two-way chamber method* is not essentially different in principle from the above, except in so far as it eliminates any gravitational gradient involved in the choice. It consists of a flat circular container connected by capillary orifices to two horizontal test-tubes (figure 2). The two test-tubes respectively contain water and an attractive solution of known strength. In this set-up the air is static. Thus circulation of air, as in devices of the 'olfactometer' type, does not complicate interpretation of results.

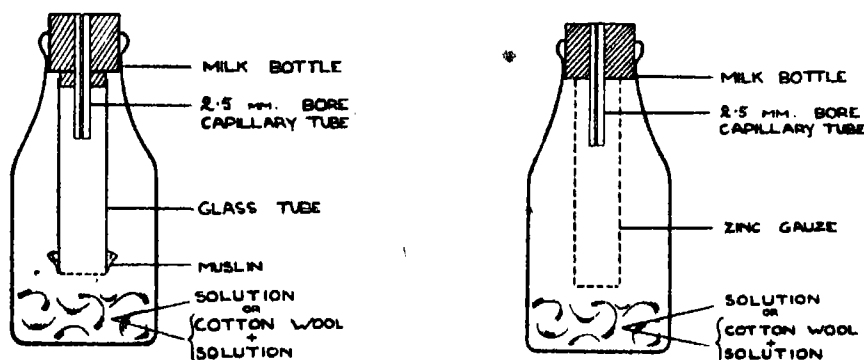


FIGURE 1. Bottle traps.

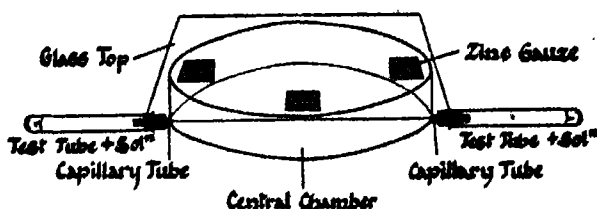


FIGURE 2. Two-way chamber.

In one respect, the procedure involved in such experiments is fundamentally different from that of the trapping method. Experiments by the trapping method involve simultaneous release of equal numbers of flies of two or more phenotypes. Tests with the two-way chamber method involve introduction of flies of one phenotype only in one and the same release chamber. For comparative purposes, an experiment of this sort therefore involves releasing flies of two or more phenotypes into two or more two-way chambers of the same dimensions at the same time in the temperature-controlled dark room. Since the release chamber of the two-way method is relatively small, a relatively high proportion of flies enter both tubes in the absence of any attractive solutions therein. Thus, the *criterion of*

indifference is an equal number of flies trapped in both tubes. The criterion of a *positive* reaction is a simultaneous excess in the experimental and deficiency in the control tubes respectively above or below the mean figure for flies trapped during the same period when neither trap contains bait. For the same reason, variability of numbers entering the tubes in successive tests is in general high, when neither trap is baited, and indeed provides almost as sensitive a measure of positive or negative response as does the *mean* itself (see table 5).

In experiments involving the trapping method, a relatively small proportion of flies get trapped when no bottles contain an attractive substance. This small proportion caught in unbaited traps remains more or less the same when half the traps are baited, but much greater numbers then enter the baited traps. If we use the term *migrants* for flies which leave the release chamber for one or other of the traps, in both the trapping and two-way chamber methods, we may contrast the two methods as follows:

method	traps	migrants	
		experimental trap	control trap
trapping	none baited	very few	very few
	half baited	many	very few
two-way chamber	neither baited	relatively many	relatively many
	one baited	many	very few

For scoring the results of the two methods we therefore adopt different procedures. We tabulate those of experiments involving the trapping method by citing the percentage of released flies of each class trapped in the bottles respectively containing and *not* containing the bait. For experiments involving the two-way chamber method, we tabulate the *olfactory index*, O_i , defined as follows. If E is the number of flies trapped in the experimental tube, i.e. the tube containing the bait, and C is the number in the control tube, containing^a water,

$$O_i = \frac{100E}{E + C}.$$

The criterion of indifference is therefore that O_i does not *significantly* differ from 50, though it may be somewhat more or less in any one trial. The criterion of a full response is that O_i does not significantly differ from 100 and that variability is relatively low.

(b) *Humidity*. For all experiments here described, the apparatus was a choice chamber of the type designed by Gunn & Kennedy (1936). Provided the sexes are kept apart, the flies do not appear to interfere with one another; so it is possible to use twenty or more animals in a single chamber. In a large number of experiments, involving some 15,000 individual observations in all, normal female flies gave no significant response to humidity. On the other hand, males showed a consistent preference for the *wet* side of the chamber when the R.H. difference was greater than 50 %. Accordingly, a standard humidity chamber was used to offer

the flies a choice between accumulating over distilled water or over sulphuric acid (density 1.84). The temperature was constant within 0.5°C of 24°C . Illumination was from above, and the chamber rotated slowly through 90° at regular intervals. A shadow passing over the chamber induced the flies to scatter. A quicker and more regular response of males occurred after previous desiccation over concentrated sulphuric acid for 3 hr. at 30°C . This procedure was therefore adopted. The index $100W \div (W + D)$ records the response as the percentage of flies found on the wet side of the chamber.

(c) *Temperature.* Preliminary experiments on normal (*Or*) flies were first carried out in several ways. The object was to find out roughly what sort of behaviour was normal to *Drosophila*. Figures 3 and 5 show two forms of apparatus used.

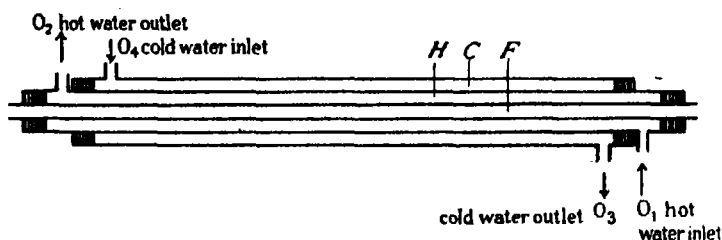


FIGURE 3. Temperature gradient tube.

(i) *Thermal gradient tube.* This consists of a straight tube (*F*) surrounded by two water-jackets (*H* and *C*). Hot water at constant temperature flows through *H* in one direction and cold water, also at constant temperature, flows through *C* in the opposite direction. The temperature in *F* is read by dragging a thermometer through the tube. By appropriate adjustments of the openings *O*₁, *O*₂, *O*₃ and *O*₄, it is possible to obtain a series of curves relating temperature to distance along the tube *F* (figure 4).

(ii) *Two-way chamber.* This is the apparatus shown in figure 5, which is self-explanatory.

Results obtained with apparatus described above are capable of more than one interpretation. Excess of flies at one or another temperature may be the result either of active choice, *sensu stricto*, or of variation with respect to activity or metabolism at different temperatures. We have therefore used, in addition to the foregoing, the T-tube apparatus shown in figures 6*a* and *b*. *T* is a T- or Y-tube drawn out at the ends to a narrow neck. It fits into a test-tube (*A*), inserted into a second test-tube (*B*). *B* fits tightly into a water-jacket (*C*). One arm of the T-tube is maintained at a higher temperature than the other by passing through the jackets hot or cold water from thermostatically controlled tanks. Flies are introduced to the apparatus at *D*. Once they enter the tube *B* they cannot get out again, and the numbers trapped on each side of the T are counted at the end of the experiment. The apparatus thus provides a true choice. The index $100C \div (C + H)$ will be used to express the number of animals on the cold side as a percentage of the total trapped.

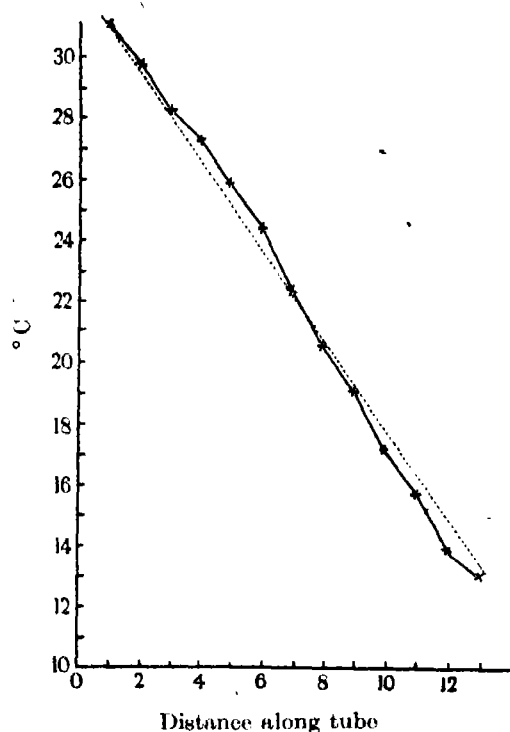


FIGURE 4. Typical temperature gradient obtained with apparatus shown in figure 3.

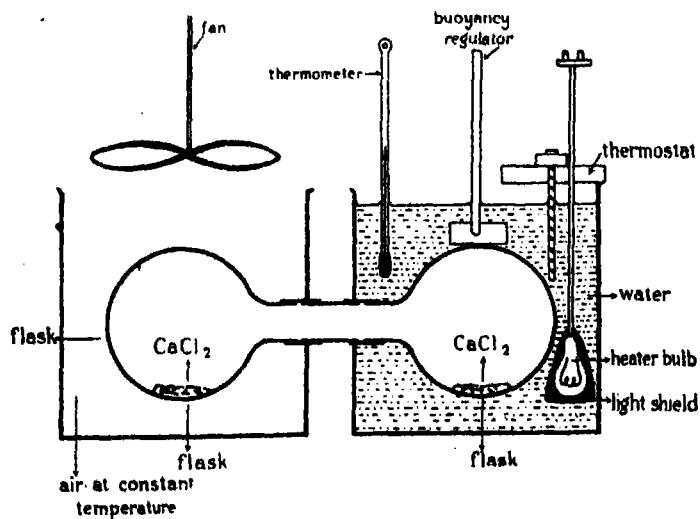
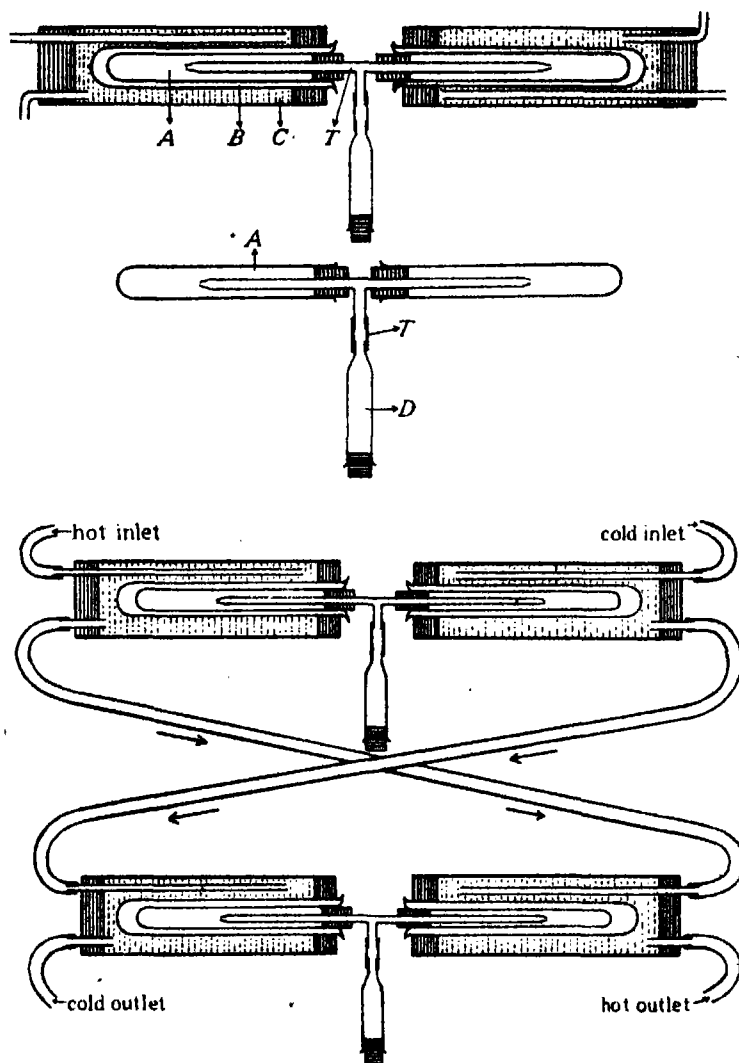


FIGURE 5. Temperature choice chamber.

FIGURE 6a,b. *T* temperature apparatus.

3. OLFACTORY RESPONSES

The results of experiments involving the two types of apparatus used for the study of reaction to chemical stimuli converge to the same general conclusions. The two-way chamber proved to be the more flexible and sensitive, and it will suffice to give a few examples representative of many involving the trapping method. Experiments of both types have to be carried out in a dark room, preferably with temperature control. The temperature of the dark room for these experiments did not vary more than a degree in the neighbourhood of 25° C. Needless to say, it was necessary to switch on the light during the short period required for scoring results.

(a) *Trapping method.* Our first table, in which *M* stands for the bait as defined in the preceding section and *W* for water, shows the percentages of the three *antennaless* phenotypes and control wild-type (*Oregon*) flies, respectively, caught in traps baited with the one or the other, together with the total number of each group released.

TABLE 1

solution	percentage of flies of each group trapped				total of each group released
	<i>A</i> ₀	<i>A</i> ₁	<i>A</i> ₂	<i>Or</i>	
<i>M</i>	1	31.5	52.5	58.7	400
<i>W</i>	3	8	5	6.7	

In any experiments of this type account must be taken of the possibility that the structural defect may involve a mechanical impediment to success in reaching the assigned goal. Accordingly, it was necessary to make observations on the three *antennaless* phenotypes when deprived of the power to reach their goal by flight. Comparison was therefore made of the reactions of the three *antennaless* phenotypes from the double recessive *antennaless-vestigial* (*ant-vg*) stock. The results of such an experiment appear in table 2.

TABLE 2

solution	percentage of flies of each group trapped			total of each group released
	<i>A</i> ₀ - <i>vg</i>	<i>A</i> ₁ - <i>vg</i>	<i>A</i> ₂ - <i>vg</i>	
<i>M</i>	4.5	46.4	98.2	112
<i>W</i>	0	1.0	0	

Experiments recorded in the two preceding tables, together with others in which flight-defective mutants were used, provide strong evidence for the following conclusions:

(i) Homozygous flies of *antennaless* stock which do not exhibit the gene, i.e. possess two normal antennae (*A*₂), behave like normal flies with respect to chemical stimuli known to attract the latter.

(ii) Homozygous flies of *antennaless* stock with unilateral exhibition, i.e. flies with only one antenna (*A*₁), show a positive response but a weaker one than flies with two antennae.

(iii) Homozygous flies of *antennaless* stock with complete exhibition, i.e. flies with no antennae (*A*₀), fail to give a response.

(iv) The weakened response of the *A*₁ phenotype and the failure of the *A*₀ phenotype to respond do not depend on any function which the antennae may conceivably have in relation to the mechanism of flight.

These conclusions do not exclude one possibility. The following question may still be asked. Do the antennae have any sensory function *vis à vis* the mechanical response of crawling into or through a narrow orifice? It is possible to investigate this by putting flies of the three phenotypes in a large tube with several capillary

outlets and illuminated from above. Since the two-way chamber method supplies a conclusive answer to the question posed above, it will not be necessary to tabulate results of experiments of this type. It suffices to state that flies of all three phenotypes find their way with equal facility through such capillary orifices when subjected to photic stimulation evoking the appropriate movements.

Table 3 shows the results of an experiment designed to test the response of *aristapedia* (*ss^a*). The control flies were of two stocks, *yellow-vermilion-forked* (*y-v-f*) and *filamentless* (*ft*). The latter is a mutant discovered by F. W. Robertson

TABLE 3

solution	percentage of flies of each group trapped			total of each group released
	<i>ss^a</i>	<i>y-v-f</i>	<i>ft</i>	
<i>M</i>	43.6	61.9	70.0	360
<i>W</i>	3.3	3.6	1.9	

and described in a forthcoming publication from this department. The characteristic which recognizably distinguishes it from wild type is merely the absence of filaments attached to the egg case of the latter. Two conclusions emerge from this table:

(i) *Aristapedia* (*ss^a*) responds positively to solutions of the type which attract wild-type flies, but apparently not so strongly as wild type. The reduced response (about 30 % below par) is statistically significant.

(ii) The structural modification of the antenna by this gene does not of itself entail abolition of the chemoreceptive function of the antenna.

From this we may draw the further conclusion that chemoreceptivity depends on microscopic elements present *both* in the normal antenna of the wild fly and in the leg-like appendage which replaces it in the mutant *aristapedia*, but absent in the normal leg.

(b) *Two-way chamber method*. Figure 7 exhibits in graphical form the results of an experiment involving the release of *antennaless* phenotypes *A₀* and *A₂* taken on the same day from the same cultures in separate chambers of the same dimensions each baited with a dilute solution (1/10) of *M* in the experimental tube and water in the control one. It is here necessary to state that a two-way chamber does not evoke a positive response from normal flies if the concentration of the bait solution exceeds a certain limit. That is the reason for using a *dilute* solution of *M*. On the assumption that there exists a preferred concentration of the exuded vapours, there are several possible physical explanations for the fact that a more concentrated solution acts as a more efficient bait when the traps are vertical and the release chamber is large. They have no apparent relevance in this context.

Response of flies with two antennae in the experiment exhibited in figure 7 is positive and striking. That of flies with no antennae is but slightly positive and not necessarily significant. The same conclusion emerges from the series of experiments summarized in table 4, which gives counts of flies trapped in both tubes at the end

of a 6 hr. test with various concentrations of a modified attractive fluid M_4 , whose composition appears below. These experiments were done in pairs (I, II and III), each pair consisting of phenotypes A_2 and A_0 taken on the same day from the same culture bottles. Two (I and II) were on flies homozygous both for the genes *antennaless* and *vestigial*, one (III) on flies homozygous for *antennaless* but with normal wings. The flies with two antennae (A_2) give a response which does not significantly depart from the criterion of a complete response; those with no antennae (A_0) give a response which does not differ significantly from the criterion of indifference. In experiments cited, there is no variation towards the lower side of the limit of indifference, but in other experiments flies with no antennae did in fact yield values of O_i with corresponding deviations in the opposite direction.

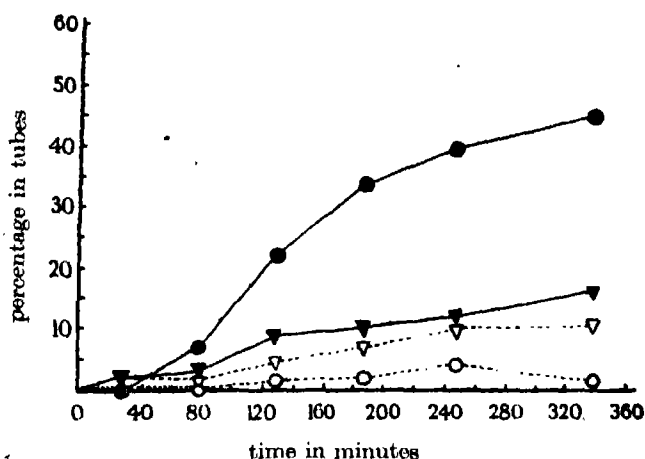


FIGURE 7. Response of A_2 and A_0 phenotypes in the two-way chamber to a supraliminal chemical stimulus. —●— A_2 in experimental tube; ---○--- A_2 in control tube; —▼— A_0 in experimental tube; ---▽--- A_0 in control tube.

TABLE 4. OLFACTORY EXPERIMENTS ON PHENOTYPES WITH TWO ANTENNAE (A_2) AND NO ANTENNAE (A_0). NON-MIGRANTS ARE ANIMALS WHICH REMAIN IN CENTRAL CHAMBER

exp.	cultures	phenotype	solution	migrants		non-migrants	O_i
				E	C		
1	antennaless vestigial (I)	A_2 -vg	M_4	55	1	44	98
2	antennaless vestigial (II)	A_2 -vg	$M_4 \times 10$	28	3	59	90
3	antennaless (III)	A_2 -vg ⁺	$M_4 \times 2$	39	5	22	89
1	antennaless vestigial (I)	A_0 -vg	M_4	12	11	77	52
2	antennaless vestigial (II)	A_0 -vg	$M_4 \times 10$	16	14	62	53
3	antennaless (III)	A_0 -vg ⁺	$M_4 \times 2$	17	17	32	50

Data from another type of experiment based on the two-way chamber method likewise discouraged the suspicion that there might be a small residual margin of chemoreceptivity in A_0 phenotypes localized in some part of the body other than

the antennae. Such experiments are worth quoting to show the variation of the olfactory index for normal flies when the bait is subliminal. In experiments on the threshold concentration it is advisable to eliminate yeast as a constituent of the bait solution because it is difficult to assess either its composition or its concentration. Preliminary tests also showed that the presence of butyric acid did not appreciably affect the results of experiments in the two-way chamber. Accordingly,

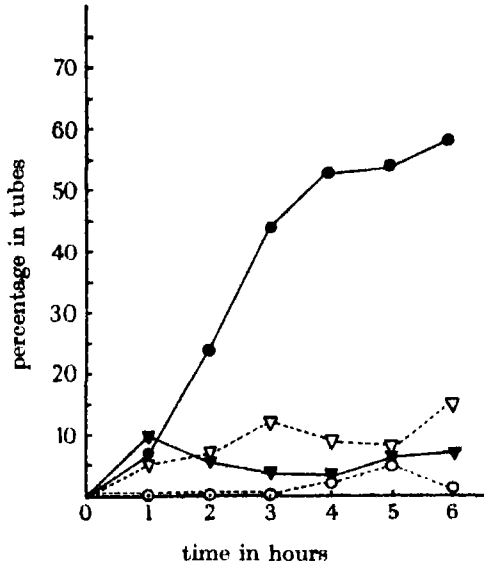


FIGURE 8. Response of normal (*ft*) flies to a supra- (—●—) and sub-liminal (—▼—) stimulus.

—●— Experimental M_4 [supra-liminal]; —▼— Experimental M_4 [sub-liminal]
 —○— Control M_4 [supra-liminal]; —▽— Control M_4 [sub-liminal]

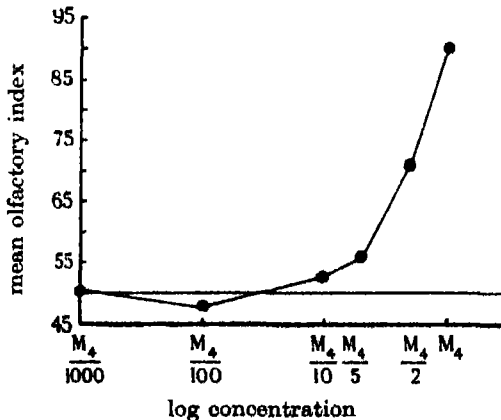


FIGURE 9. Stimulation threshold for *ft* flies.

our threshold experiments are based on dilutions of a mixture (M_4) of the following constituents in the concentrations specified:

M_4	
acetic acid	0.01 %
ethyl alcohol	0.16 %
acetaldehyde	0.00001 %
ethyl acetate	0.00001 %
methyl acetate	0.00001 %
distilled water	to 100 %

Table 5 and figures 8 and 9 summarize the data of a series of experiments involving several successive determinations of the olfactory index for each concentration of M_4 in the baited tube. They illustrate both the variation of the mean index on either side of 50 at subliminal concentrations and, as might be expected, an increase of the coefficient of variation in the neighbourhood of the threshold. The fact that a fly may sometimes escape from the trap explains the occasional drop in the curves shown in fig. 8. There is little need to emphasize the fact that

TABLE 5. OLFACTORY THRESHOLD OF FILAMENTLESS

concentration	mean (O_i)	C.O.V. sample	concentration	mean (O_i)	C.O.V. sample
M_4	90 \pm 4	10	$M_4/100$	48 \pm 9	42
$M_4/2$	71 \pm 10	27	$M_4/1000$	51 \pm 12	48
$M_4/5$	56 \pm 8	28	M_4/∞	50 \pm 8	33
$M_4/10$	53 \pm 11	45			

the conclusions which emerge from tests with the two-way chamber are entirely consonant with those which emerge from tests with the trapping method in so far as they refer to the effect of presence or absence of antennae in homozygous flies of the *antennaless* stock. It will suffice to cite the results of a series of tests on *aristapedia* (ss^a) to show that the results of the two methods again point to the same conclusion. These results appear summarized in table 6.

TABLE 6. OLFACTORY INDEX FOR *ARISTAPEDIA* (ss^a)

concentration	mean (O_i)	C.O.V. sample
M_4	85.6 \pm 2	5.3

4. HUMIDITY RESPONSES

Table 7 gives the response of normal (Oregon) undesiccated males to a range of humidities. There appears to be a preference for an R.H. of about 90 %. It will be seen that a wet reaction is always obtained when the R.H. difference exceeds about 50 %. Table 8 shows results for desiccated males and for desiccated females in the *standard* humidity gradient.

TABLE 7

theoretical R.H. %		$\frac{100W}{W+D}$	$\frac{d}{\sigma_d}$	number of position records
D	W			
100	100	45.9	1.46	630
87	100	46.3	1.28	600
75	100	48.6	0.47	580
67	100	54.6	1.57	580
45	100	51.8	0.62	600
26	100	68.1	6.86	672
0	100	67.2	7.37	864
0	87	78.0	10.57	600
0	75	69.3	6.83	580
0	67	66.0	6.13	600
0	45	54.3	1.69	630
0	26	53.3	1.14	600
0	0	52.5	0.89	640

TABLE 8

sex	$\frac{100W}{W+D}$	$\frac{d}{\sigma_d}$	number of position records
males	69.9	12.19	2424
females	51.5	0.54	651

In no experiment did we observe a clear avoiding reaction at the boundary. Animals which are moving from one side to the other do so without hesitation. However, males tend to remain much more quiescent on the wet than on the dry side of the chamber. This is not true of females, which move freely throughout the chamber. Even males previously desiccated at 30° C, and extremely active when first introduced to the chamber, settle down rapidly. It thus seems that the humidity reactions of the males are due rather to an inhibition of movement than to a *taxis*.

Table 9 is a comparison between the response of *aristapedia* (*ss^a*) males, desiccated for 3 hr. at 30° C over concentrated sulphuric acid, and that of *Oregon* males as shown in table 8.

TABLE 9

stock	$\frac{100W}{W+D}$	number of position records	$\frac{d_{(a-b)}}{\sigma_{d(a-b)}}$
(a) <i>Or</i>	69.9	2424	15.50
(b) <i>ss^a</i>	89.6	1200	

There is clearly a greater response of *ss^a* as compared with *Oregon*. The reason for this will be discussed when we consider identification of the sensillae responsible. Meantime, it suffices to state that observations on *aristapedia* tally with others on *Oregon* in so far as they show no clear-cut avoiding reaction. The flies become rapidly quiescent on the wet side of the chamber.

Table 10 shows humidity responses of males of the three *antennaless* phenotypes desiccated also over sulphuric acid, and of both females and males of the same stocks, taken from saturated air.

TABLE 10

phenotype	sex	treatment	$\frac{100W}{W+D}$	$\frac{d}{\sigma_d}$	number of position records
A_2	male	desiccated	60.8	3.79	600
A_1	male	desiccated	57.5	2.34	580
A_0	male	desiccated	30.42	10.86	1420
A_0	male	undesiccated	39.7	3.73	640
A_0	female	undesiccated	36.7	4.69	600

The results shown in this table indicate that the absence of antennae does not abolish but modifies the humidity response. It leads to a reaction opposite to that characteristic of normal animals. That is to say, *antennaless* flies show a *negative* reaction to high relative humidities. This suggests that two sets of antagonistic reactions are being dealt with. One set involves impulses from the antennae, resulting in a positive response to high relative humidities. Another set involves impulses from elsewhere, resulting in negative response to high relative humidities. Since desiccation slightly *increases* the dry response of A_0 animals and also *increases* the wet reaction of A_2 individuals, it is possible that one is dealing not with two different types of receptors, but the same type of receptor and different types of innervation. The normal response can thus be regarded as inhibition of a basic dry reaction. This is of some interest in view of the fact that most insects show a negative reaction to high relative humidities (Gunn *et al.*).

5. TEMPERATURE

Figure 10 shows preliminary experiments on the distribution of normal (*Or*) flies in temperature gradients from 22.5 to 34.5° C. Clearly there is accumulation at the lower end of the temperature scale, but this result is probably emphasized by a disposition to gather at the *ends* of a tube even in the absence of a temperature gradient. Nevertheless, the results are striking, and have been confirmed by an alternative technique. Table 11 shows results obtained by use of *Or* flies in the choice chamber. As before, there is a large excess of flies on the cooler side of the apparatus. Experiments, such as those described above, roughly indicate the normal behaviour of *Drosophila* to temperature differences, but they are ambiguous for a reason already mentioned (p. 6). The T apparatus (described on p. 6) is not equally open to this objection and is therefore preferable. Table 12 shows a set of typical results for the various stocks concerned. Experiments summarized in table 12 show that normal (*Or*) flies do not react to high temperatures unless above 31° C. In the neighbourhood of 41° C the reaction is strong. Homozygous flies of *antennaless* stock with two antennae (A_2) or no antennae (A_0) both respond strongly to such a temperature. From this we may at least conclude that thermoreceptors

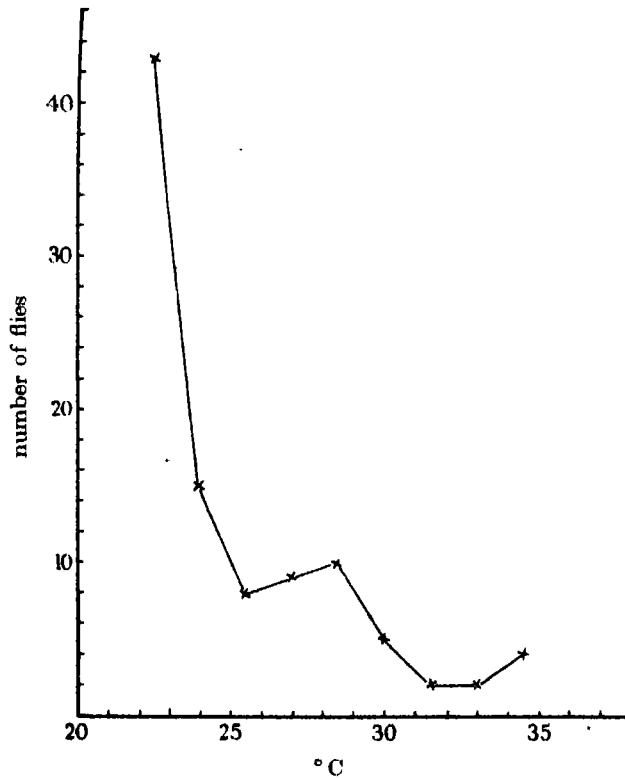
FIGURE 10. Distribution of normal (*Or*) flies in a linear temperature gradient.

TABLE 11

sex	temperature		response 100 × cold cold + hot	number of animals	stock
	cold ° C	hot ° C			
female	23.5	33	85.0	40	<i>Or</i>
female	23.5	34	85.0	40	<i>Or</i>
female	22	27.5	77.5	40	<i>Oph</i> (extra control stock)

TABLE 12

temperature ° C		index 100C H + C	d σ _d	stock	sex
hot	cold				
31.5 ± 0.5	19.0	50.0	0	<i>Or</i>	male
41.5 ± 2	20.0 ± 1	98.2	17.3	<i>Or</i>	male
41.5 ± 2	20.0 ± 1	95.9	13.5	<i>Or</i>	female
41.0 ± 1	18.5	97.4	13.03	<i>A</i> ₁	male
41.0 ± 1	18.5	84.7	13.6	<i>A</i> ₁	female
42.0 ± 2	19 ± 0.5	81.1	11.2	<i>A</i> ₀	male
42.0 ± 2	19.0 ± 0.5	80.4	10.6	<i>A</i> ₀	female
41.0 ± 1	19.5 ± 0.5	42.4	2.67	<i>se</i> ^a	female

are not *confined* to the antennae, although they may occur there. Homozygous flies of *aristapedia* stock show no reaction to this temperature. On the face of it this fact is puzzling. Its implications will be discussed in connexion with histological observations below. We may here note that the occurrence of R.H. differences does not affect the conclusions stated. *Antennaless* (A_0) animals normally show a *dry* reaction, and would therefore tend to accumulate on the *warmer* side of the gradient, if the reaction described were due to humidity. It is true that *ss^a* and male *Or* flies have wet reactions, but females of *Or* stock show no humidity reactions. Moreover, *ss^a* males do not in fact accumulate on the wetter (cold) side of the T apparatus.

6. RELATION OF CHEMORECEPTIVITY TO MATING BEHAVIOUR IN *DROSOPHILA*

Investigations are being carried out to determine the relations of various sense organs to the mating behaviour of *D. melanogaster*. Here one is concerned only with the part played by the chemoreceptors. *Antennaless* (A_0) flies mate freely in the dark. It is therefore evident that neither photoreceptivity nor chemoreceptivity plays an *essential* part in the mating of *D. melanogaster* under laboratory conditions. Experiments show that long-distance olfactory stimuli originating in the female play *no* part in assisting the male to reach her. Long-distance olfactory stimuli emanating from the male likewise play no detectable part in the search by the female for the male.

Table 13 shows the results of releasing flies of Oregon stock in a trapping chamber, when the bait offered was an individual of opposite sex. The experimental traps contained either 100 males or 100 females each, and a small muslin-covered tube of water. Controls contained water only. These experiments fail to confirm the supposition that males are attracted to females or females to males by olfactory stimuli. Similar results were obtained with the use of the two-way chamber

TABLE 13

number of flies released (T)	sex of flies in baits	numbers trapped		$\frac{100E}{T}$	$\frac{100C}{T}$
		E	C		
100 males	males	11	13	11	13
100 males	females	2	6	2	6
200 males	males	7	5	3.5	2.5
200 males	females	10	5	5	2.5
200 females	(a) males	(a) 2	4	(a) 1.0	2
	(b) females	(b) 5		(b) 2.5	

method. From these and other similar experiments, it may be concluded that the only function of the chemoreceptors *vis à vis* mating behaviour of *D. melanogaster* under field conditions is to attract both males and females to one and the same food patch. In so far as they do play a part in mating, the role of the chemoreceptors

is to ensure the concentration of individuals of both sexes in sufficient propinquity to provide opportunities of sexual congress evoked by stimuli other than volatile by-products of metabolism.

7. HISTOLOGICAL OBSERVATIONS

The data summarized in table 14 are based on examination of sections of etherized imagos, fixed in Kahle's fluid at 60° C, dehydrated successively with ethyl and normal butyl alcohol (Begg & Sang 1944). The stains used were gentian violet and light green.

TABLE 14

stock	olfactory response	humidity response	temperature response	putative sense organs of antennae
<i>Or</i>	++	+	++	pit with thin-walled pegs; thin-walled cones and pegs; trichoid sensillae
<i>ss^a</i>	+	++	0	thin-walled cones and pegs; trichoid sensillae
<i>A₀</i>	0	—	++	—



FIGURE 11. Sections through the long axis of the distal joint of the antenna of Oregon flies, showing pits and surface sensillae. Magnification $\times 230$.

Figure 11 shows sections through the long axis of the distal joint of the antenna of an Oregon fly. Three main types of putative sense organs occur therein:

- (i) Thin-walled sensillae sunk in a pit near the base of the funiculus.
- (ii) Fine thin-walled surface and pegs and cones distributed over the surface of the antenna.
- (iii) Thick-walled trichoid sensillae, commonly assumed to be receptive to tactile stimuli.

The *pit organs* (i) are absent in *aristapedia* (*ss^a*), but the other types of sensillae are present. In *aristapedia*, however, the thin-walled cones are thinner than those of normal (Oregon) flies. Since *aristapedia* responds to both sorts of stimulation in their absence, it is clear that the pit organs are essential neither to chemoreceptivity nor to hygrosensitivity. It is therefore possible that the thin-walled cones and pegs fulfil both functions; but there may well be differentiation of function between cones and pegs, as also between individual sensillae of either or

both types. If both types perform both functions, one would expect intensified reaction to humidity to accompany greater sensitivity to chemical stimuli. This is not so. *Aristapedia* gives a greater humidity, but a somewhat diminished olfactory, response. The issue is complicated by the fact that *aristapedia* has no pit organs. Flüggé (1934) suggested that the latter are long-distance chemoreceptors. Hence diminution of response in their absence would be expected. This is, in fact, what is found. Thus a hypothesis which accords best with our data is as follows:

(1) The pit organs on the funiculus are long-distance chemoreceptors, more sensitive than the surface pegs and cones, but not essential to chemoreceptivity.

(2) The surface pegs or the cones, or both, are chemoreceptors.

(3) The surface cones are hygroreceptors, their finer structure in *aristapedia* accounting for the increased humidity response of this mutant.

We have not found any histological differences to account for lack of temperature response in *aristapedia* or for its presence in *antennaeless* (A_0). In this connexion, all we can say is that the antennae are not essential to the recognition of temperature differences. Sensillae occur all over the body. For all we know to the contrary, any or all of them may be temperature receptors. Some of them doubtless function as humidity receptors, initiating a reaction opposite to that governed by the antennal hygroreceptors. Our own experiments do not entitle us to distinguish one from the other. But the failure of *ss^a* to respond indicates that this gene has multiple effects.

We wish to express our thanks to Mr H. M. Packer for valuable technical assistance.

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Experimental chemotherapy of typhus

Anti-rickettsial action of *p*-sulphonamidobenzamidine and related compounds

By C. H. ANDREWES, F.R.S., HAROLD KING, F.R.S. AND JAMES WALKER

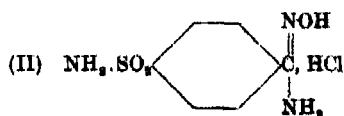
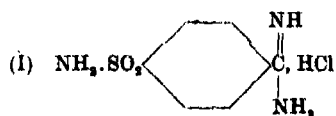
National Institute for Medical Research, Hampstead

(Received 21 December 1944)

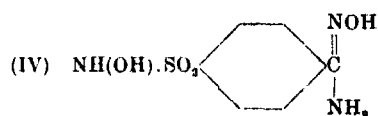
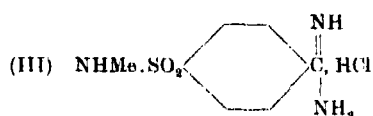
The discovery of the pronounced anti-rickettsial activity of *p*-sulphonamidobenzamidine on experimental typhus infections in mice has led to a search for compounds of enhanced therapeutic action. A large number of compounds have been prepared in development of the sulphonamido-amidine structure, but anti-rickettsial activity has been found to be confined to about a dozen substances intimately related to *p*-sulphonamidobenzamidine, only one, namely, *p*-sulphonamidobenzamidoxime, having a very slightly superior therapeutic action.

The significance of the discovery of yet another sulphonamide type with pronounced biological activity is discussed.

When this investigation was begun no substances were known with a chemotherapeutic action on experimental typhus infections. Andrewes, King & van den Ende (1943) had made a determined attempt to develop a chemotherapy of the virus diseases, influenza and vaccinia, but without success, and on reviewing the situation it was thought that an attack on experimental typhus might be more profitable, since the rickettsiae responsible for this disease, although intracellular parasites like the viruses, are considered by many to stand between the viruses and bacteria. Many of the compounds tried on virus diseases were tested on an experimental murine typhus infection in mice without revealing any noticeable therapeutic activity. A request was then made, in view of the urgency of this problem, to the Therapeutic Research Corporation of Great Britain and to Imperial Chemical Pharmaceuticals Ltd. for a list of compounds available for trial against small intracellular parasites, and from this list a selection was made by us of compounds which we deemed worthy of test on rickettsial infections. Among the substances chosen there was one, *p*-sulphonamidobenzamidine hydrochloride (I), described by Boots Pure Drug Co., Levene & Pyman (1942) and later by Delaby & Harispe (1943), which had an unmistakable therapeutic action on experimental typhus infections in mice. The biological aspects of the investigation have been described recently by Andrewes, King, van den Ende & Walker (1944), and the present account is limited to certain chemical aspects.

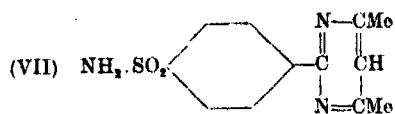
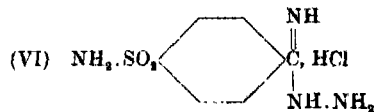
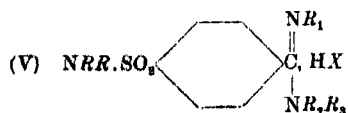


p-Sulphonamidobenzamidine hydrochloride (I) is a simple substance reminiscent of sulphanilamide. It was confidently hoped that it would, like sulphanilamide, lend itself to development in numerous ways with production of substances showing enhanced activity. The contrary, in fact, has proved to be the case. Anti-rickettsial activity is confined to a few compounds of closely related structure, of which *p*-sulphonamidobenzamidoxime hydrochloride (II) is the most important. This substance as base or hydrochloride proved, if anything, to be a little more active than the corresponding amidine (I). When substituents were introduced into the sulphonamide radicle common to these two substances, there was complete suppression of all activity with the exception of *p*-sulphonmethyldiamidobenzamidine hydrochloride (III) and *p*-sulphonhydroxylamidobenzamidoxime (IV).



In both of these the activity was reduced. Introduction of higher alkyl groups such as butyl, of dialkyl groups such as dimethyl, diethyl and di- β -hydroxyethyl, led to suppression of activity. The same was observed on introduction of the phenyl, *p*-sulphonamidophenyl and *p*-sulphondimethylamidophenyl groups. In the sulphanilamide series the incorporation of heterocyclic nuclei into the sulphonamide group has led to the important drugs, sulphapyridine, sulphathiazole, sulphamethylthiazole and sulphadimethylpyrimidine, but the incorporation of similar heterocyclic nuclei into the sulphonamide group of the amidine (I) or the amidoxime (II), where experimentally realized, has led to suppression of anti-rickettsial action in the tolerable doses. The *meta*-isomeride of (I), *m*-sulphonamidobenzamidine hydrochloride, also proved to be inactive.

Substitution in the amidine group of *p*-sulphonamidobenzamidine (I) also had a dystherapeutic effect. Thus *p*-sulphonamidobenz-N-methylamidine hydrochloride (V, $\text{RR}_2\text{R}_3 = \text{H}$; $\text{R}_1 = \text{Me}$; $\text{X} = \text{Cl}$) and *p*-sulphonamidobenz-NN'-dimethylbenzamidine hydrochloride (V, $\text{RR}_2 = \text{H}$; $\text{R}_1\text{R}_3 = \text{Me}$; $\text{X} = \text{Cl}$), both of which arose by the action of methylamine on *p*-sulphonamidobenziminoethyl ether hydrochloride, were inactive.

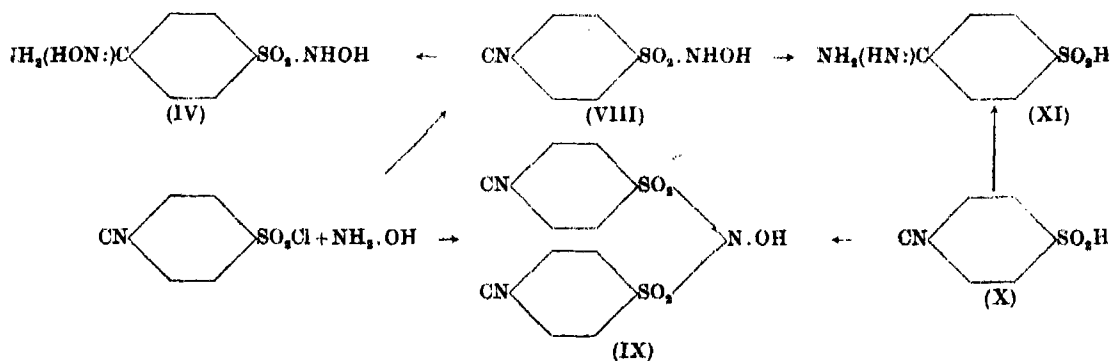


By the action of ammonia, methylamine or dimethylamine on the iminochloride from *p*-sulphondimethylamidobenzmethylamide, the three amidines *p*-sulphondimethyl-

amidobenz-N-methylamidine picrate (V, $RR_1 = \text{Me}$; $R_2R_3 = \text{H}$; $X = \cdot\text{C}_6\text{H}_3\text{O}_7\text{N}_3$), *-NN'-dimethylamidine hydrochloride* (V, $R_2 = \text{H}$; $RR_1R_3 = \text{Me}$; $X = \text{Cl}$) and *-NNN'-trimethylamidine picrate* (V, $RR_1R_2R_3 = \text{Me}$; $X = \cdot\text{C}_6\text{H}_3\text{O}_7\text{N}_3$) were obtained. The second of these was inactive and the others were not examined further in view of the dystherapeutic effect of the sulphonamidylamide group in the series with an unsubstituted amidine group.

In *p-sulphonamidobenzamidrazone hydrochloride* (VI) no anti-rickettsial activity was found and none in 2-*p-sulphonamidophenyl-4:6-dimethylpyrimidine* (VII) prepared by the action of *p-sulphonamidobenzamidine* base on acetylacetone. When, however, *p-sulphonamidobenzamidoxime* was converted into *p-sulphonamidobenzureideoxime* by the action of cyanic acid, considerable anti-rickettsial activity was retained.

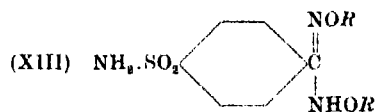
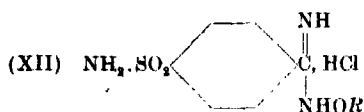
Since activity was exhibited by *p-sulphonhydroxylamidobenzamidoxime* (IV) it was cogent to develop this type of structure. This substance had arisen unexpectedly during an attempt to prepare *p-cyanobenzenesulphonhydroxylamide* (VIII) by the action of *p-cyanobenzenesulphonyl chloride* on excess of hydroxylamine, addition of hydroxylamine taking place at the nitrile group simultaneously with the reaction at the sulphonyl group. By limiting the amount of hydroxylamine to one molecular proportion, *p-cyanobenzenesulphonhydroxylamide* was obtained together with *NN-di-p-cyanobenzenesulphonhydroxylamide* (IX), the constitution of which was confirmed by its synthesis by the action of nitrous acid on *p-cyanobenzenesulphinic acid* (X).



When an attempt was made to convert *p-cyanobenzenesulphonhydroxylamide* (VIII) into the corresponding amidine, amidine-formation took place with internal scission of the sulphonhydroxylamide group and production of *p-sulphinobenzamidine* (XI), a weakly basic substance which did, however, form a *hydrochloride* but was devoid of anti-rickettsial activity. A similar scission was observed in an attempt to prepare the amidoxime from *p-cyanobenzenesulphonhydrazide*, the product of the action of hydroxylamine on this substance being *p-sulphinobenzamidoxime*. This scission of the sulphonhydroxylamide and sulphonhydrazide groups is in keeping with the known tendency of such substances to form sulphinic acids, although

the reagents used in the present examples are somewhat unusual. *p*-Sulphino-benzamidine (XI) was obtained alternatively by the direct route from *p*-cyanobenzenesulphinic acid (X), and in a similar way *p*-sulphobenzamidine, a high-melting, sparingly soluble, weakly basic substance, was obtained from potassium *p*-cyanobenzenesulphonate.

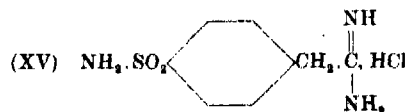
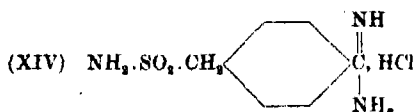
Although *p*-sulphonhydroxylamidobenzamidine has not been prepared, its *O*-methyl, *O*-ethyl, *O*-propyl and *O*-butyl ethers have been, as well as the corresponding series of *sulphon-O*-alkylhydroxylamidobenzamidoximes. They were all therapeutically inactive. When, however, *O*-alkylation was effected in the amidoxime group of (II) with an unsubstituted sulphonamide group in the *para*-position, some activity was observed. For the preparation of these substances direct *O*-alkylation of benzamidoxime-*p*-sulphonamide (II) was not successful. They were accessible, nevertheless, by the action of excess of the *O*-alkylhydroxylamines on *p*-sulphonamidobenziminoethyl ether hydrochloride in alcoholic solution. In this way *p*-sulphonamidobenzamidoxime-*O*-methyl, -*O*-ethyl, -*O*-propyl and -*O*-butyl ethers were obtained in some cases as the *bases* and in all cases as the *hydrochlorides* (XII). During the preparation of the three lower homologues, it was possible to isolate from the reaction mixture the *dialkoxybenzamidine-p*-sulphonamides (XIII), a new group of weakly basic substances hitherto apparently only represented by the parent substance dihydroxybenzamidine (Ley 1898) and its monobenzyl ether (Ley & Ulrich 1914).



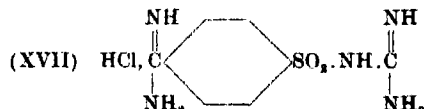
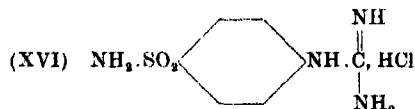
The *methyl* and *ethyl* ethers of type (XII) and the *dimethyl*, *diethyl* and *dipropyl* ethers of type (XIII) showed some anti-rickettsial activity, but quantitatively less than that of the parent amidoxime.

The influence of homologation on the amidine (I) and the amidoxime (II) is capable of experimental development in various ways. When a methyl group was introduced into the *ortho*-position to the sulphonamide group with formation of 4-sulphonamido-3-methylbenzamidine hydrochloride, the activity was just detectable, but the corresponding 4-sulphonamido-3-methylbenzamidoxime hydrochloride was slightly more active. The preparation of the isomeric 4-sulphonamido-2-methylbenzamidine was not successful since 2-cyano-5-sulphonamidotoluene, like many *ortho*-substituted cyanobenzenes, failed to undergo imino-ether formation under the usual conditions. This nitrile was prepared by the Sandmeyer reaction on 2-amino-5-sulphonamidotoluene which itself was obtained through the action of chlorosulphonic acid on aceto-*o*-toluidide. The Russian authors Goldyrev & Postovskii (1938) assumed that the sulphonic acid group entered the *para*-position to the methyl group during this reaction, but the alternative position *para* to the acetamido group seems preferable.

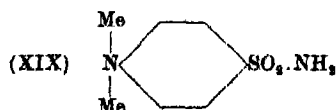
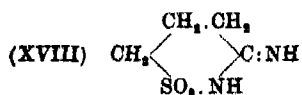
In two other homologues, *p*- ω -sulphonamidotolamidine hydrochloride (XIV) and *p*-sulphonamidophenylacetamidine hydrochloride (XV), the sulphonamide and amidine groups respectively are separated from the nucleus by methylene groups. This change was accompanied by a loss of anti-rickettsial activity.



The effect of replacing an amidine group by a guanidine group was next explored, since King, Lourie & Yorke (1937, 1938) had shown that in a series of long-chain $\omega\omega'$ -diguanidines activity against trypanosomes was retained when the guanidine groups were replaced by amidine or isothiourea groupings. *p*-Sulphonamidophenylguanidine hydrochloride (XVI) was obtained by the action of cyanamide on sulphanilamide hydrochloride, but the product proved to be devoid of activity. Sulphaguanidine is a useful drug in the sulphanilamide series; its counterpart in the present series *p*-guanylbzenzenesulphonylguanidine hydrochloride (XVII) was prepared through *p*-cyanobenzenesulphonyl-*S*-methylisothiourea obtained by the action of *p*-cyanobenzenesulphonyl chloride on *S*-methylisothiourea; it was also found to be inactive. This observation again confirms the dystherapeutic effect of substituents in the sulphonamide group. The direct action of *p*-cyanobenzenesulphonyl chloride on guanidine base under the conditions which yield acetyl sulphaguanidine (Bratton, Marshall, White & Litchfield 1940) gave NN'-di-*p*-cyanobenzenesulphonylguanidine $(\text{CN} \cdot \text{C}_6\text{H}_4 \cdot \text{SO}_2\text{NH})_2\text{C}:\text{NH}$, an acidic substance which formed sparingly soluble sodium and potassium salts.



The results so far recorded show convincingly that the anti-rickettsial activity of the parent sulphonamidobenz-amidine and -amidoxime is a highly specific property which is reduced by quite minor alteration of these structures and is lost by further modification. It was, however, possible that activity might be possessed by structures showing wider departure from the present types. An aliphatic analogue of sulphonamidobenzamidine, namely ζ -sulphonamido α -enanthamidine hydrochloride, $\text{SO}_2\text{NH}_2 \cdot (\text{CH}_2)_6 \cdot \text{C}(:\text{NH})\text{NH}_2$, HCl , was prepared without difficulty, but when an attempt was made to prepare a still simpler amidine from γ -cyanopropanesulphonamide, the novel product was *anhydro*- γ -sulphobutyramidine (XVIII).



*N*⁴-Dimethylsulphanilamide (XIX) and *p*-methylsulphonylbenzamidine hydrochloride (XX) show a formal resemblance to *p*-sulphonamidobenzamidine hydrochloride (I). From the former the more soluble *methochloride* was prepared, and for comparison with this its homologue *p*-sulphonamidobenzyltrimethylammonium chloride. All of these substances proved inactive on experimental typhus; *p*-methylsulphonylbenzamidine hydrochloride (XX) has, however, been found to be a valuable curative agent against experimental gas-gangrene infections (Evans, Fuller & Walker 1944).

A large number of amidines and amidoximes devoid of sulphonamide groups and of activity have been prepared and tested in a similar manner; those which are new are described in the experimental portion of this communication and a complete list of all the substances tested on experimental typhus will be recorded elsewhere.

DISCUSSION

The following table gives a list of the active compounds discovered in this investigation, together with their relative activities. The tests were carried out by intranasal inoculation of mice with a 1 : 10⁶ dilution of a stock rickettsial suspension 2 hr. after the first injection of the drug. Further injections were given on the morning and afternoon of the second and third days and on the morning of the fourth day. The mice were autopsied 1 week after infection, the complete suppression of lesions in the lungs in each of six mice being taken as the end point.

TABLE OF RELATIVE TOXICITIES AND ACTIVITIES

	A	B
	mg.	mg.
<i>p</i> -Sulphonamidobenzamidine hydrochloride	24	4
<i>p</i> -Sulphonamidobenzamidoxime hydrochloride	24	2
<i>p</i> -Sulphonamidobenzamidoxime	16	4
<i>p</i> -Sulphonmethylamidobenzamidine hydrochloride	16	8
<i>p</i> -Sulphonhydroxylamidobenzamidoxime	16	4
<i>p</i> -Sulphonamido- <i>m</i> -methylbenzamidoxime hydrochloride	16	> 8
<i>p</i> -Sulphonamidobenzamidoxime- <i>O</i> -methyl ether hydrochloride	16	8
<i>p</i> -Sulphonamidobenzamidoxime- <i>O</i> -ethyl ether hydrochloride	8	4
<i>NN'</i> -Dimethoxybenzamidine- <i>p</i> -sulphonamide	8	4
<i>NN'</i> -Diethoxybenzamidine- <i>p</i> -sulphonamide	8	> 8
<i>NN'</i> -Dipropoxybenzamidine- <i>p</i> -sulphonamide	16	8
<i>p</i> -Sulphonamidobenzureideoxime	8	2
<i>p</i> -Sulphonamido- <i>m</i> -methylbenzamidine hydrochloride	8	> 4

A = Maximum tolerated dose for 15 g. mouse (intraperitoneal injection).

B = Minimal effective (wholly suppressive) dose given twice daily.

Sparingly soluble bases were given suspended in 5% gum-acacia in saline.

It will be apparent from this table and the preceding account that no substances were found with greater activity than the two simple unsubstituted parent substances, and that activity is reduced or disappears with the slightest modification of

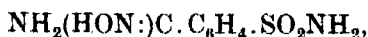
these structures. The question now arises whether a study of the structures of the active and inactive substances described in this communication affords any clue as to their mode of action. Before this can be discussed it is necessary to emphasize the difficult type of biological system with which we are dealing.

The simplest systems of host and parasite capable of chemotherapeutic study are those of bacterial and protozoal infections in general, where the infecting organism leads an extracellular parasitic existence within the host. Of still greater complexity are plasmodial infections in birds where there is a cellular (tissue) phase as well as a blood phase, and most difficult of all from the chemotherapeutic point of view are virus and rickettsial infections where there is an intracellular infection and where the metabolism of the parasite is probably intimately related to that of the host cells. Most of the drugs which have been developed for the treatment of blood infections with protozoa or bacteria act directly on the invading infective agent. In the case of rickettsial (and virus) infections the possibility is ever present that the drug may either act directly on the parasite or on some constituent of the invaded host cell upon which the rickettsiae depend for their nutrition; this material may be the special metabolites elaborated by the cell or the enzymic structures concerned in their production. In viruses, certainly, nucleic acids are major functional constituents, and the work of Schoenheimer and his colleagues (Barnes & Schoenheimer 1943; Plentl & Schoenheimer 1944) has shown that the nitrogen of purines and pyrimidines, the fundamental building blocks of nucleic acids, is derived in the cell from ammonia and not from large nitrogenous molecules, although the concession is made that pyrimidines may be built up partially in the cell from larger units than ammonia. In the case of the rickettsiae, it is also very probable that the nucleic acids are essential, and it may be that the necessity for an intracellular habitat for both viruses and rickettsiae is due to their dependence upon the nucleic acid-synthesizing abilities of the cell.

The advent of sulphanilamide has been a landmark in chemotherapeutic theory and practice. Woods (1940) discovered that the action of sulphanilamide on bacteria was inhibited by *p*-aminobenzoic acid, and the further disclosure that this acid was an essential metabolite for bacteria has led to the view that, owing to the pictorial resemblance of sulphanilamide and *p*-aminobenzoic acid, there is a competitive inhibition of *p*-aminobenzoic acid by sulphanilamide in the bacterial cell leading to bacteriostasis and finally to death. Another sulphonamide drug introduced by Domagk (1942) for the treatment of anaerobic infections is marfanil or *p*-sulphonamidobenzylamine, $\text{NH}_2\text{CH}_2\cdot\text{C}_6\text{H}_4\cdot\text{SO}_2\text{NH}_2$; this is not antagonized by *p*-aminobenzoic acid. To account for the bacteriostatic effect of this drug, Schreus (1942) has suggested the existence of a natural growth factor, *p*-aminomethylbenzoic acid, $\text{NH}_2\text{CH}_2\cdot\text{C}_6\text{H}_4\cdot\text{CO}_2\text{H}$, with which the drug enters into competitive inhibition. Evans, Fuller & Walker (1944) have discovered that *p*-methylsulphonylbenzamidine, $\text{NH}_2(\text{HN}:)\text{C}\cdot\text{C}_6\text{H}_4\cdot\text{SO}_2\text{CH}_3$, and *p*-methylsulphonylbenzylamine,



are also very effective therapeutic agents against gas-gangrene organisms, and in the present communication it has been shown that *p*-sulphonamidobenzamidine, $\text{NH}_2(\text{HN:})\text{C} \cdot \text{C}_6\text{H}_4 \cdot \text{SO}_2\text{NH}_2$, and *p*-sulphonamidobenzamidoxime,



are effective against rickettsiae *in vivo*. Clearly, to postulate special growth factors, with which each of these types can enter into competitive inhibition, as Schreus has done for marfanil, is untenable, and some other mechanism for their mode of action must be sought.

All the above substances are of comparatively simple molecular build and all contain the sulphonyl group linked to carbon, $\text{>C} \cdot \text{SO}_2$ —. With the sole exception of dimethylsulphone, Me_2SO , found by Ruzicka, Goldberg & Meister (1940) in dried ox blood and by Pfiffner & North (1940) in ox adrenals, this group is foreign to the animal body. Mann & Keilin (1940) found that the enzyme carbonic anhydrase, a protein, containing zinc in its prosthetic group, is specifically inhibited at high dilutions by the unsubstituted sulphonamide group in benzenesulphonamide, $\text{C}_6\text{H}_5 \cdot \text{SO}_2\text{NH}_2$. In this sulphonamide and in other nuclear substituted sulphonamides, as, for example, sulphanilamide, the sulphonamide group has the specific power of adding on to some unknown structure in the enzyme and thus affecting its activity. It is excluded that carbonic anhydrase is the enzyme inhibited by all the above-mentioned sulphonyl-containing drugs, but it is conceivable and possible that different enzymes might be inhibited by each of these drug types with a special mechanism for the sulphanilamide group.

There is, however, another alternative which needs brief discussion. Is it not possible that all these sulphonyl drugs of relatively small molecular size gain entrance into the parasitic organism and during reproduction, when active synthesis is going on, become built up into structures through combination with the non-sulphonyl portion of the molecule and thus produce a vital structure which can no longer function normally owing to the foreign nature of the sulphonyl group? Such a view, in our opinion, would not be inconsistent with the competitive inhibition between sulphanilamide and *p*-aminobenzoic acid, since the *p*-aminobenzoic acid may normally be utilized synthetically through its amino-group. The building up of sulphanilamide through its amino-group into a complex by a competitive process would naturally disrupt the normal functioning of the vital structure; the experiments which have been described in the literature on the reversibility of the inhibition are not inconsistent with this view. In this connexion, it is significant that a free amino-group is essential in all drugs derived from sulphanilamide for activity, and it is difficult to believe that this is merely used for salt formation. Blanchard (1941) has, indeed, found that in yeast part of the *p*-aminobenzoic acid is bound, with the masking of the amino-group. The notion that *p*-aminobenzoic acid is built up into a larger complex as part of its normal function is not new. Poth & Knotts (1942) took the view that *p*-aminobenzoic acid may combine with a hypothetical substance *X* to form a metabolic enzyme necessary for bacterial metabolism, whilst Mietzsch

(1942) suggested that *p*-aminobenzoic acid is built up into a polypeptide. If the latter involves the carboxyl group as well, it would explain the inability of the sulphonamide group to continue the polypeptide structural sequence. On this view *p*-sulphonamidobenzamidine or *p*-sulphonamidobenzamidoxime might be built up into some essential vital structure through the amidine or amidoxime group. The elements of either of these groups, namely, N-C-N, enter twice into every purine and once into every pyrimidine, essential constituents of all nucleic acids. Although Schoenheimer's work suggests that the purines and pyrimidines are built up in the cell from ammonia, it does not preclude the use of small molecular substances which have gained entry into the cell from being incorporated into such structures during the periods of intense synthesis. In this connexion it is significant that the rickettsiae are not harmed by these drugs *in vitro*, but only when they are undergoing reproduction *in vivo* when nucleic acid synthesis must be active. Whether this synthesis is taking place inside the rickettsia, which is larger than the majority of viruses, or outside the rickettsia but inside the cell of its host, is difficult to determine, but in view of Schoenheimer's work, the former view is preferable.

Although purine and pyrimidine formation has been used to picture the way in which the amidine and amidoxime groups could be utilized in complex building, it should not be overlooked that these groups are respectively imino- and hydroxy-imino-amide groups, and in cells might undergo conversion into the amide for structure building. *p*-Sulphonamidobenzamide was tested in this connexion but proved inactive. There is, therefore, a specific quality in the amidine group which is not its strongly basic nature, since the amidoxime is equally active, but less basic, and the dialkoxamidines are also active, but very weakly basic. None of the active substances recorded in the table have structures incompatible with cellular incorporation into pyrimidines and purines during the reproductive phase with its accompanying intense nucleic acid synthesis.

Finally, the active substances recorded in this communication have been discovered by essentially a process of trial and error. The hypothesis of competitive inhibition has not been applied, and as a means of finding new chemotherapeutic agents may only be of value where the essential growth factors for the organism whose activities it is desired to curb are already known. In this connexion it is interesting to note that McKinsty & Reading (1944) in attempting the development of a rational approach to the chemotherapy of virus diseases have examined the effect of a large number of synthetic pyrimidines on the course of experimental murine poliomyelitis in the hope that compounds of this type might compete with the normal virus pyrimidine components for the synthetically active enzyme systems involved; unfortunately, they attained no clear-cut success.

When the work described in this communication was practically complete three other communications appeared in which accounts are given of other drugs with some action on experimental or clinical typhus. Moragues, Pinkerton & Greiff (1944) found that penicillin given in large doses reduced the mortality in experimental murine typhus, whilst Peterson (1944) demonstrated that 'forbisen' and

toluidine blue possess therapeutic activity against the Wilmington strain of murine typhus. Yeomans, Snyder, Murray, Zarafonitis & Ecker (1944) have, quite recently, claimed that *p*-aminobenzoic acid has a favourable action in typhus in man, a clinical investigation based on some unpublished experiments with typhus in mice by Snyder, Maier & Anderson (1942).

The two most active drugs in our series were tested clinically on typhus infection in man, but did not give encouraging results (compare Andrewes, King, van den Ende & Walker 1944). A full report of the clinical trials will be published later.

EXPERIMENTAL

Amidines not containing sulphur

3:4-Piperonylamidine hydrochloride. Piperonylic acid (8.3 g.) was heated with phosphorus pentachloride (10.4 g.) for 1 hr. on the boiling water-bath. On removing the oxychloride, the residue, in benzene, was added portionwise with shaking to ammonia solution (50 c.c., $d_{40} 0.88$) at $0-5^{\circ}$. The amide (7.35 g.) was collected and crystallized from absolute alcohol (75 c.c.), yield 6.2 g., m.p. 167° . This was treated with phosphorus pentachloride (7.6 g.) and heated on the boiling water-bath for 15 min. The oxychloride was pumped off and the crystalline residue of piperonylonitrile crystallized from 25 % aqueous alcohol (525 c.c.), yield 5.3 g., m.p. 94° .

A mixture of this nitrile (3.67 g.) with absolute alcohol (2.9 g.) and benzene (5 c.c.) was saturated with dry hydrogen chloride at 0° and kept at 0° for a few days. The solvent was removed at room temperature under diminished pressure, and the residual solid added to saturated alcoholic ammonia solution (150 c.c.) and kept at 37° for 3 days. The clear solution was evaporated to dryness, a crop of ammonium chloride being removed at an intermediate stage. The final residue was mixed with a little water, unchanged piperonylonitrile (0.5 g.) being removed by ether, and, on concentration of the aqueous solution to a small volume, 3:4-piperonylamidine hydrochloride separated as a white chalky crystalline mass composed of minute needles, yield 2.09 g. It was crystallized from an equal weight of water and had m.p. $240-241^{\circ}$. (Found: C, 48.0; H, 4.5; N, 14.0. $C_8H_8O_2N_2$, HCl requires C, 47.9; H, 4.5; N, 14.0%.)

***p*-Bromobenzamidine hydrochloride.** *p*-Bromobenzonitrile (9.05 g.), purified by sublimation, was suspended in absolute alcohol (5.8 g.) and benzene (5 c.c.) and the solution saturated with hydrogen chloride at 0° . The process of amidine formation was then carried on as described above. The alcoholic ammonia solution was evaporated to dryness, the residue taken up in water and unchanged nitrile (2.1 g.) removed by ether. The aqueous solution on concentration gave *p*-bromobenzamidine hydrochloride (8.3 g.). It was crystallized from 3.5 parts of boiling water and separated in long flat plates (8.07 g.), m.p. $264-265^{\circ}$. (Found: C, 31.1; H, 4.6; N, 10.2; H_2O , 13.1. $C_7H_5N_2Br$, HCl, $2H_2O$ requires C, 30.9; H, 4.5; N, 10.3; H_2O , 13.3%.)

***p*-Carbamylbenzamidine hydrochloride.** *p*-Aminobenzamide (9.1 g.) was submitted to the Sandmeyer reaction, using cuprous cyanide at 80° , and gave a crude mixture

of *p*-chlorobenzamide and *p*-cyanobenzamide which on crystallization from water (200 c.c.) gave *p*-cyanobenzamide (4.0 g.). The solid was recovered from the aqueous mother liquor, and on fractional sublimation at 160° and 15 mm. gave *p*-chlorobenzamide (1.15 g. confirmed by mixed m.p.), and at 200–220° and 15 mm. gave *p*-cyanobenzamide (1.35 g.).

p-Cyanobenzamide crystallizes from 40 parts of boiling water in needles, m.p. 222°. (Found: N, 19.1. $C_8H_6ON_2$ requires N, 19.2 %). Pfannl & Dafert (1912), by heating terephthalic acid diureide to 360° at 20 mm., obtained a substance to which they gave this composition and described it as crystals but did not record a m.p. The constitution of their product is queried in Beilstein's *Handbuch* (1932).

The nitrile (3.65 g.) suspended in absolute alcohol (2.5 mol.) was converted into the amidine in the usual way. After completely removing the ammoniacal solvent, the residue was fractionally crystallized from water, when nitrile (1.9 g.) was recovered unchanged and *p*-carbamybenzamidine hydrochloride (1.49 g.) obtained as the more soluble component. This amidine hydrochloride crystallizes in prismatic needles, m.p. 302–303°. (Found: C, 44.3; H, 5.4; N, 19.0; H_2O , 8.4. $C_8H_6ON_3 \cdot HCl \cdot H_2O$ requires C, 44.1; H, 5.6; N, 19.3; H_2O , 8.3 %.)

A repetition of this preparation confirmed the difficulty of imino-ether formation.

3-Amino-4-hydroxybenzamidine dihydrochloride. *p*-Cyanophenol (9.0 g.) in glacial acetic acid (27 c.c.) was run into a stirred mixture of nitric acid (9.0 g., *d* 1.42) and glacial acetic acid (9.0 g.) kept at 50° (compare Cousin & Volmar 1914). Reaction did not usually take place immediately, but when it was over the solution was poured into water and the solid which separated (10.8 g.), m.p. 143°, collected. It was crystallized from 9 vol. of absolute alcohol, yield 10.4 g., m.p. 144°. Borsche (1917) gives m.p. 144–145° for *o*-nitro-*p*-cyanophenol.

O-Nitro-*p*-cyanophenol (13.2 g.) in spirit (85 c.c.) was treated with a solution of hydrated stannous chloride (80 g.) in aqueous hydrochloric acid (160 c.c., 16 %) and heated on the water-bath for 5 hr. The clear solution was diluted with water, the tin removed as sulphide and the filtrate evaporated to dryness. The residual solid (yield 7.2 g.) was crystallized from absolute alcohol. *o*-Amino-*p*-cyanophenol hydrochloride crystallizes in tiny plates from alcohol and has no definite m.p. (Found: C, 49.0; H, 4.1; N, 16.3. $C_7H_6ON_2 \cdot HCl$ requires C, 49.3; H, 4.1; N, 16.4 %.) A sample of base was recovered from the hydrochloride; it had a m.p. in agreement with that recorded in the literature.

The aminocyanophenol hydrochloride (4.2 g.) was suspended in a mixture of equal parts (each 10 c.c.) of absolute ethyl alcohol and dioxan, and amidine formation carried through as usual. The ammoniacal alcohol solution was evaporated to dryness and the residue treated with 3*N*-hydrochloric acid until the solution was acid to litmus. On concentration under reduced pressure 3-amino-4-hydroxybenzamidine dihydrochloride (2.84 g.) separated. It was crystallized from *N*/10-hydrochloric acid (4 c.c.) and separated in columns, m.p. 292° (efferv.). (Found: C, 32.6; H, 6.1; H_2O , 14.3. $C_7H_6ON_3 \cdot 2HCl \cdot 2H_2O$ requires C, 32.3; H, 5.8; H_2O , 13.9 %.)

Nicotinamidine hydrochloride. Nicotinonitrile (3 g.) (McElvain & Goese 1941) was converted to nicotinimino ether hydrochloride (Karrer, Schwartzenbach, Benz & Solmssen 1936) which was then set aside with 8 % alcoholic ammonia for 5 days at room temperature. Alcohol and excess ammonia were distilled off, and the white solid residue digested with hot alcohol (80 c.c.) and filtered hot from ammonium chloride. The filtrate was concentrated (to about 30 c.c.) and again filtered hot from traces of ammonium chloride. On cooling, a copious separation of sheaves of lance-shaped crystals took place (1.9 g.), m.p. 189–190°. (Found: C, 46.1; H, 5.2; N, 26.4. Calc. for $C_6H_7N_3$, HCl: C, 45.7; H, 5.1; N, 26.7 %.) Barber & Slack (1944) have also recently described this substance, prepared by a slightly different method. The properties recorded are in agreement with the above.

Amidoximes not containing sulphur

3:4-Piperonylamidoxime hydrochloride. Hydroxylamine hydrochloride (3.5 g.) was dissolved in water (20 c.c.), anhydrous sodium carbonate (2.5 g.) added and finally piperonylonitrile (7.35 g.) with ethyl alcohol (30 c.c.). The solution was boiled under reflux for 24 hr. and the alcohol distilled off. Treatment of the semi-solid aqueous residue with a large volume of ether removed unchanged nitrile (0.8 g.) and a small quantity of amidoxime base which was recovered by extraction of the ether with a little 2N-hydrochloric acid. The base was recovered from this by addition of sodium bicarbonate and mixed with the main crop present in the semi-solid aqueous residue (above). The total base was collected (6.4 g.) and crystallized twice from absolute ethyl alcohol, yield 4.9 g., m.p. 164–165°. *3:4-Piperonylamidoxime* is soluble in 7.5 vol. of boiling absolute alcohol and crystallizes therefrom in bundles of plates. (Found: C, 53.6; H, 4.4; $C_8H_8O_3N_2$ requires C, 53.3, H, 4.4 %.) For the preparation of the *hydrochloride*, the base (1.8 g.) was dissolved in 2N-hydrochloric acid (5 c.c.), and on keeping the hydrochloride separated in prisms, m.p. 204–205° (efferv.). (Found: C, 44.3; H, 4.3; N, 12.8. $C_8H_8O_3N_2$, HCl requires C, 44.3; H, 4.2; N, 12.9 %.)

p-Chlorobenzamidoxime hydrochloride. *p*-Chlorobenzonitrile (6.85 g.) and equivalent proportions of anhydrous sodium carbonate and hydroxylamine hydrochloride were mixed in water (10 c.c.) and alcohol (30 c.c.) and boiled for 20 hr. The alcohol was removed and the residue made alkaline with sodium bicarbonate and thoroughly extracted with ether. The ethereal solution was freed from amidoxime by extraction with 10 % hydrochloric acid and on evaporation left unchanged nitrile (0.6 g.). The *amidoxime base* was again liberated from the acid solution and crystallized from its own weight of absolute alcohol, as clusters of tablets, yield 4.55 g., m.p. 134–135°. (Found: C, 49.6; H, 4.3. $C_7H_7ON_2Cl$ requires C, 49.3; H, 4.1 %.)

The *hydrochloride* separated from an acidified solution of the base in large hexagonal plates, m.p. 185° (efferv.). (Found: C, 37.6; H, 4.5. $C_7H_7ON_2Cl$, HCl, H_2O requires C, 37.3; H, 4.5 %.) A sparingly soluble by-product was found to be *p*-chlorobenzamide, m.p. 176–177°. (Found: C, 54.2; H, 3.8. Calc. for C_7H_6ONCl : C, 54.0; H, 3.9 %.)

p-Bromobenzamidoxime hydrochloride. *p*-Bromobenzonitrile, freshly sublimed (9.1 g.), was boiled with hydroxylamine (1 mol.) in aqueous alcohol for 20 hr. On removal of the alcohol, the solution, with an oily layer, was extracted with ether, leaving a crystalline hydrochloride (0.47 g.) undissolved, and having m.p. 263°, unchanged on recrystallization. It gave a base, m.p. 178°, but attempts to identify it were unsuccessful. The main ethereal solution after extraction with 16 % hydrochloric acid gave on evaporation a mixture of *p*-bromobenzonitrile (0.7 g.) and *p*-bromobenzamide (0.7 g.) separated by subliming-out the former. The hydrochloric acid extract readily gave *p*-bromobenzamidoxime hydrochloride (9.0 g.), which crystallized in large tablets, and a small quantity of *p*-bromobenzamide (0.4 g.). The amidoxime hydrochloride on recrystallization had m.p. 202° (efferv.). (Found: C, 31.4; H, 3.7; N, 10.4. $C_7H_7ON_2Br$, HCl, H_2O requires C, 31.2; H, 3.7; N, 10.4 %.)

p-Bromobenzamidoxime was liberated from the hydrochloride by addition of aqueous sodium bicarbonate and required 50 vol. of water for its recrystallization, from which it separated in long needles, m.p. 144–145°. (Found: C, 39.4; H, 3.1; N, 12.8. $C_7H_7ON_2Br$ requires C, 39.1; H, 3.3; N, 13.0 %.)

Amidines containing the groups $—SO_2NH_2$, $—SO_2NHR$, $—SO_2NRR'$ and $—SO_2C<$

p-Cyanobenzenesulphonamide. Sulphanilamide (172 g.; 1 g.-mol.) was warmed with concentrated hydrochloric acid (200 c.c.) and water (330 c.c.). The clear solution was cooled to 0° giving a thick sludge of the hydrochloride, into which was stirred, slowly, an aqueous solution (200 c.c.) of sodium nitrite (69 g.) until an immediate positive starch-iodine reaction was obtained. The diazo-suspension was added moderately rapidly to a solution of technical hydrated nickel chloride (237 g.) and technical sodium cyanide (245 g.) in water (1650 c.c.) heated and stirred vigorously in a boiling water-bath, when a brisk effervescence occurred which controlled the rate of the addition of the diazonium salt. Heating and stirring were continued for 10 min. after the nitrogen effervescence ceased and the mixture was then cooled below room temperature. The crude product was collected, washed with water, drained thoroughly and then extracted with acetone. The filtered acetone extract was evaporated and the crude reddish brown crystalline residue crystallized from water (norit), affording long needles of the nitrile (123 g., 67–68 % yield), m.p. 165–166°.

Miller, Sprague, Kissinger & McBurney (1940) have used cuprous cyanide in this preparation, but we have found it inferior to the nickel salt, the product by the copper method being very difficult to purify.

p-Sulphonamidobenzamidine hydrochloride (I). *p*-Cyanobenzenesulphonamide (14.85 g.) was added to absolute alcohol (45 c.c.) and the solution saturated at 0° with dry hydrogen chloride. The reaction mixture was kept at 0° for a week, the alcohol removed at room temperature and the crystalline imino-ether hydrochloride added to saturated alcoholic ammonia (200 c.c.) and kept for a week at 37°. The solid

was collected and the filtrate evaporated to dryness. On treatment of the combined solids with a little water to remove ammonium chloride, crude *p*-sulphonamidobenzamidine hydrochloride was left (yield 14.8 g.), and on concentration of the aqueous ammonium chloride mother liquor further amidine hydrochloride (1.7 g.) was obtained with unchanged parent nitrile (0.15 g.). *p*-Sulphonamidobenzamidine hydrochloride hemihydrate is readily soluble in less than its own volume of boiling water and separates in tablets, m.p. 250–251°. (Found: C, 34.3; H, 4.7; N, 17.3; H₂O, 3.9. C₇H₆O₂N₃S, HCl, $\frac{1}{2}$ H₂O requires C, 34.3; H, 4.5; N, 17.2; H₂O, 3.7 %.) Delaby & Harispe (1943) describe the salt as anhydrous with m.p. 242°. When the hydrochloride in water was treated with the calculated amount of *N*-sodium hydroxide, the base separated in plates, m.p. 228° (efferv.). (Found: C, 42.2; H, 4.7. Calc. for C₇H₆O₂N₃S: C, 42.2; H, 4.6 %.) On attempting to recrystallize this base from water, it was converted into the diamide. On precipitating the base with ammonia, Delaby & Harispe describe the base as transparent lamellae, m.p. 251°, but repetition of their mode of preparation confirmed the m.p. 228° given above.

In some preparations of this amidine from the nitrile, the free base separated as such from the alcoholic ammonia solution.

m-Sulphonamidobenzamidine hydrochloride. *m*-Cyanobenzenesulphonamide (7.28 g.) suspended in alcohol (6.0 c.c.) and dioxan (10 c.c.) was converted into the amidine in the usual way. The ammoniacal solution was filtered from ammonium chloride which had separated and, on partial concentration, gave *m*-sulphonamidobenzamidine hydrochloride (4.65 g.) which, crystallized from water (3.5 c.c.), gave the pure salt in plates, m.p. 248–249°. (Found: C, 35.7; H, 4.1; N, 17.8. C₇H₆O₂N₃S, HCl requires C, 35.7; H, 4.3; N, 17.8 %.) A further quantity of this salt was obtained from the mother liquors with a small crop of unchanged *m*-cyanobenzenesulphonamide (0.5 g.).

p-Sulphonamidotolamidine hydrochloride (XIV). The nitrile required for the preparation of this amidine was obtained* as follows. *p*-Cyanobenzyl bromide (19.6 g.) (Case 1925) and sodium sulphite heptahydrate (25.2 g.) were refluxed with water (50 c.c.) for half an hour, when the nitrile rapidly dissolved. On cooling, sodium *p*-cyanotoluene-*o*-sulphonate (22.5 g.) separated as a microcrystalline solid, m.p. about 309°. (Found: on solid dried at 110° *in vacuo*, N, 6.7; S, 14.2. C₈H₆O₃NSNa requires N, 6.4; S, 14.6 %.) This sodium salt (21.5 g.) was mixed intimately with finely powdered phosphorus pentachloride (20.7 g.) and moistened with a few drops of phosphorus oxychloride. After 3 hr. at 75–80° on the water-bath, the mixture was added to ice-water, when an oil separated which eventually solidified. It was collected, washed with water and then shaken with 16 % aqueous ammonia solution (160 c.c.). The required *p*-cyanotoluenesulphonamide (11.1 g.) was collected after 12 hr.; it separated from 50 % alcohol in microscopic short laths, m.p. 216°. (Found: C, 48.6; H, 4.4; N, 14.1. Calc. for C₈H₆O₂N₂S: C, 49.0; H, 4.1; N, 14.3 %.) Miller *et al.* (1940) record m.p. 216–217° for this substance prepared by an alternative method.

For the preparation of the tolamidine, a suspension of the preceding nitrile (5.75 g.) in dioxan (20 c.c.) and absolute ethyl alcohol (12 c.c.) was saturated at 0° with hydrogen chloride and the process completed as usual. The clear ammoniacal solution was evaporated to dryness. The residue was extracted with cold water (40 c.c.) when unchanged nitrile (1.97 g.) remained undissolved. The filtrate was evaporated to dryness, and the residual solid (4.5 g.) was crystallized from equal parts of methyl alcohol and ethyl acetate. *p*-*ω*-Sulphonamidotolamidine hydrochloride separated in clusters of fine colourless rods, m.p. 253–254°. (Found: C, 38.5; H, 5.1; N, 16.5. $C_8H_{11}O_2N_3S$, HCl requires C, 38.5; H, 4.8; N, 16.8 %.)

p-Sulphonamidophenylacetamidine hydrochloride (XV). Into *p*-toluenesulphonyl chloride (57 g.), heated and stirred under reflux in an oil bath at 170°, bromine (18 c.c.) was dropped at such a rate that it did not accumulate in the reaction mixture. Hydrogen bromide was copiously evolved. At the end of the reaction the product was fractionally distilled giving (i) unchanged *p*-toluenesulphonyl chloride (18.2 g.), b.p. 150° at 18 mm., and (ii) *ω*-bromotoluenesulphonyl chloride (45 g.), b.p. 160–170° at 3 mm. The latter product solidified readily and could be crystallized from ligroin when short prisms separated, m.p. 72–73°. (Found: C, 31.2; H, 2.2; Cl + Br, 43.5. $C_7H_6O_2S$ ClBr requires C, 30.8; H, 2.5; Cl + Br, 42.8 %.)

The *p*-sulphonamidophenylacetonitrile required for amidine formation was obtained in poor yield from either the preceding *p*-*ω*-bromo or from *ω*-chlorotoluenesulphonyl chloride. In a typical experiment, *p*-*ω*-chlorotoluenesulphonyl chloride (8.1 g.) (Friedländer 1913) in acetone solution (25 c.c.) was mixed and shaken with 2*N*-aqueous ammonia (40 c.c.). Heat was evolved and a crystalline solid separated. The solid was collected at the end of an hour and at once treated with sodium cyanide (2 g.) in boiling 50 % aqueous alcohol (40 c.c.) for 3 hr. After acidification to Congo paper, the mixture was left overnight at room temperature, then decanted from gum which had separated, and evaporated to small bulk under reduced pressure until crystals separated. Two crystallizations from water afforded long, colourless, silky, flattened needles of *p*-sulphonamidophenylacetonitrile, m.p. 184°. (Found: C, 48.9; H, 4.1; N, 14.5. $C_8H_8O_2N_3S$ requires C, 49.0; H, 4.1; N, 14.3 %.)

The above nitrile (7 g.) was dissolved in absolute alcohol (30 c.c.) and dioxan (75 c.c.), and the process of amidine formation carried through in the customary way. The alcoholic ammonia solution was evaporated to dryness and the residue extracted with a small volume of water, when a small residue (0.7 g., m.p. 182–184°) of unchanged nitrile remained undissolved. The aqueous solution was evaporated to dryness and the syrupy residue gradually crystallized. *p*-Sulphonamidophenylacetamidine hydrochloride was extremely soluble in water and the lower alcohols, and separated from methyl alcohol-ethyl acetate (1:1) in fine colourless prisms, m.p. 215°. (Found: C, 38.6; H, 4.8. $C_8H_{11}O_2N_3S$, HCl requires C, 38.5; H, 4.8 %.)

4-Sulphonamido-3-methylbenzamidine hydrochloride. Aceto-*m*-toluidide (116 g.) was submitted to the same treatment as that described below for the *ortho*-derivative. The free aminosulphonamide (73.3 g.) separated from spirit in colourless, microscopic

rhombs, m.p. 170–171°. Goldyrev & Postovskii (1938) record m.p. 168°. When applied to the amine (54.7 g.), the conditions for the Sandmeyer reaction, described above for sulphanilamide, afforded 3-cyanotoluene-6-sulphonamide (31.3 g.) which separated from dilute aqueous alcohol in long narrow rectangular plates, m.p. 195° with prior sintering. (Found: C, 48.8; H, 4.3; N, 14.3. $C_8H_8O_2N_2S$ requires C, 49.0; H, 4.1; N, 14.3 %.)

A solution of the above nitrile (8 g.) in absolute alcohol (20 c.c.) and dioxan (60 c.c.) was saturated with hydrogen chloride and the process of amidine formation carried through. The ammoniacal alcohol solution deposited a heavy crystalline solid (6.9 g.) which was collected and washed with a small volume (14 c.c.) of water. The substance was resuspended in water and carefully neutralized with *N*-hydrochloric acid to pH 6. Evaporation of the solution to dryness and crystallization of the product from water gave 4-sulphonamido-3-methylbenzamidine hydrochloride, colourless trapezoidal tablets, m.p. 257–258°. (Found: C, 38.6; H, 5.0; N, 16.6. $C_8H_{11}O_2N_3S$, HCl requires C, 38.5; H, 4.8; N, 16.8 %.)

2-Amino-5-sulphonamidotoluene. Aceto-*o*-toluidide (83 g.) was converted to the chlorosulphonyl derivative with chlorosulphonic acid (396 c.c.) in the usual manner. The sulphonyl chloride was collected and treated, while still damp, with 16 % aqueous ammonia (500 c.c.) and left overnight. Aqueous sodium hydroxide (60 c.c., 50 % solution) was added and the solution, after filtration, was treated with excess of saturated ammonium sulphate solution. The precipitated sulphonamide was collected and immediately deacetylated by being refluxed with 16 % aqueous hydrochloric acid (500 c.c.) for an hour. The free amine, liberated by careful addition of alkali and finally bicarbonate, was collected (63.8 g.) and crystallized from 40 % alcohol and then from spirit, when colourless prisms (54 g.) separated, m.p. 174°.

Goldyrev & Postovskii (1938) ascribe a different orientation to this substance and record m.p. 176°. A small amount (4.5 g., m.p. 135–147°) of a lower-melting by-product was isolated from the mother liquor.

2-Cyano-5-sulphonamidotoluene. The preceding amine (54 g.) was submitted to the Sandmeyer reaction, using the conditions described above for sulphanilamide. The resulting nitrile separated from 40 % aqueous alcohol in microscopic rhombs (39.4 g., 69 % yield), m.p. 187°. (Found: C, 48.7; H, 4.2; N, 14.0. $C_8H_8O_2N_2S$ requires C, 49.0; H, 4.1; N, 14.3 %.)

This nitrile failed to undergo reaction with ethyl alcohol and hydrogen chloride under the usual conditions for imino-ether formation and was recovered unchanged.

p-Sulphonmethylamidobenzamidine hydrochloride (III). *p*-Acetamidobenzenesulphonyl chloride (23.36 g.), finely powdered, was suspended in dry ether (100 c.c.), and alcoholic methylamine solution (20 g., 33 %) added in portions with cooling. After keeping for 12 hr. the ether was distilled, the residue treated with water and the solid collected, ground with saturated sodium bicarbonate solution, filtered and washed with water, yield 17.05 g. One part of *p*-acetamidobenzenesulphonmethylamide required 20 parts of boiling water to dissolve it, and it crystallized therefrom in needles, m.p. 184–185°. (Found: N, 12.6. Calc. for $C_9H_{12}O_3N_2S$: N, 12.3 %.)

Mangini (1940) gives m.p. 152–153° and crystalline form, platelets, but we have not been able to consult the original paper. The main bulk of crude material was hydrolysed by boiling with 16 % hydrochloric acid (100 c.c.) for 1 hr. and the base liberated by alkali, yield 12 g. For analysis, one part of base required 30 vol. of boiling water for recrystallization. *p*-Aminobenzenesulphonmethylamide crystallized in silky needles, m.p. 110°. (Found: C, 45.4; H, 5.5; N, 15.2. Calc. for $C_7H_{10}O_2N_2S$: C, 45.1; H, 5.4; N, 15.1 %.)

The foregoing substituted aniline (10.35 g.) was diazotized in hydrochloric acid solution and added to a solution of cuprous cyanide (4.97 g.) in water (40 c.c.) containing sodium cyanide (5.4 g.) at 50°. The solid which separated was collected and extracted (Soxhlet) with ether, yield 7.6 g. It was crystallized from water (300 c.c.) and then from its own weight of boiling ethyl acetate, yield 4.83 g. *p*-Cyanobenzenesulphonmethylamide crystallized in plates, m.p. 127–128°. (Found: C, 48.9; H, 4.0; N, 14.2. $C_8H_8O_2N_2S$ requires C, 48.9; H, 4.1; N, 14.3 %.)

The foregoing nitrile (4.5 g.) in absolute ethyl alcohol (11.5 c.c.) was converted into the imino-ether hydrochloride and then into the amidine in the usual way. On concentration of the alcoholic ammonia solution, crude amidine hydrochloride was obtained in three crops which on crystallization from water gave *p*-sulphonmethylamidobenzamidine hydrochloride (3.4 g.), m.p. 290–291°. This salt was soluble in 1.5 vol. of boiling water and crystallized in small rhombs. (Found: C, 38.3; H, 5.0; N, 16.6. $C_8H_{11}O_2N_3S$, HCl requires C, 38.5; H, 4.9; N, 16.8 %.)

p-Sulphondimethylamidobenzamidine hydrochloride. *p*-Aminobenzenesulphondimethylamide (19.8 g.) in water (60 c.c.) and concentrated hydrochloric acid (20 c.c.) was diazotized at 0–5° with sodium nitrite (7.2 g.) in water (72 c.c.). The solution was added slowly to one of cuprous cyanide (8.8 g.) in water (80 c.c.) containing sodium cyanide (9.6 g.) at 0–5°. When all had been added, the temperature was raised to 50° to complete the reaction. An ethereal extract of the cooled reaction mixture gave crude nitrile (19.0 g.) which on crystallization from 5 vol. of absolute ethyl alcohol gave *p*-cyanobenzenesulphondimethylamide, yield 16.45 g., m.p. 124°, as clusters of plates. (Found: C, 51.7; H, 4.8; N, 13.1. $C_9H_{10}O_2N_2S$ requires C, 51.4; H, 4.8; N, 13.3 %.)

The above nitrile (4.2 g.) in absolute alcohol (12 c.c.) was converted into the imino-ether and then into the amidine in the usual way. The alcoholic ammonia solution was evaporated to dryness and the residue dissolved in a little water. By careful fractionation a very soluble salt (2.6 g.) was obtained free from ammonium chloride, and on crystallization from half its weight of hot water gave *p*-sulphondimethylamidobenzamidine hydrochloride, tablets, yield 2.04 g., m.p. 251–252°. (Found: C, 41.3; H, 5.6; N, 16.2. $C_8H_{13}O_2N_3S$, HCl requires C, 41.0; H, 5.4; N, 15.9 %.)

p-Sulphondiethylamidobenzamidine hydrochloride. *p*-Cyanobenzenesulphonyl chloride (11.7 g.), finely powdered, was suspended in dry ether (100 c.c.) and diethylamine (10.3 c.c.) added fairly rapidly with external cooling. After standing for 48 hr. the ether was distilled, the residue treated with water and the solid (12.6 g.) collected. It was crystallized from its own weight of absolute alcohol and *p*-cyanobenzene-

sulphondiethylamide separated in aggregates of large plates (11.4 g.), m.p. 94–95°. (Found: C, 55.5; H, 6.0; N, 11.8. $C_{11}H_{14}O_2N_2S$ requires C, 55.4; H, 5.9; N, 11.8 %.)

The above nitrile (4.96 g.) in absolute alcohol (6 c.c.) was converted into the imino-ether hydrochloride and then into the amidine in the usual way. The ammoniacal alcoholic solution was evaporated to dryness and an aqueous solution (3.0 c.c.) of the residue was made faintly acid to litmus by addition of hydrochloric acid. The crude *p-sulphondiethylamidobenzamidinium hydrochloride* (5.1 g.) separated in tablets and on crystallization from its own weight of water was analytically pure with m.p. 232–233°, yield 4.7 g. (Found: C, 45.5; H, 6.3; $C_{11}H_{17}O_2N_3S$, HCl requires C, 45.3; H, 6.2 %.)

p-Sulphonbutylamidobenzamidinium hydrochloride. To *p-acetamidobenzenesulphonyl chloride* (11.7 g.), suspended in dry ether (100 c.c.), was added *n*-butylamine (7.3 g.) with external cooling. After keeping for 24 hr. the ether was distilled, the residue treated with water and the *p-acetamidobenzenesulphonbutylamide* collected, yield 12.6 g. Recrystallization from 2.5 vol. of boiling ethyl acetate gave square plates, m.p. 139–140°. (Found: C, 53.6; H, 6.9; N, 10.6. $C_{12}H_{18}O_3N_2S$ requires C, 53.3; H, 6.7; N, 10.4 %.)

The above diamide (12.2 g.) was boiled with hydrochloric acid (80 c.c., 16 %) for 1 hr., cooled and *p-aminobenzenesulphonbutylamide hydrochloride* collected, yield 13.95 g. It crystallized from 17 parts of boiling alcohol in pearly leaflets, m.p. about 230°. (Found: C, 45.3; H, 6.2; N, 10.5. $C_{10}H_{16}O_2N_2S$, HCl requires C, 45.3; H, 6.5; N, 10.6 %.)

The above aniline hydrochloride (11 g.) was diazotized in the usual way and the diazo-solution slowly dropped into a vigorously stirred solution, kept at 50°, of cuprous cyanide (3.7 g.) and sodium cyanide (4.08 g.) in water (30 c.c.). When cold the solid was collected, and when dry was extracted with ether (Soxhlet), yield 8.9 g. It was crystallized first from 70 % alcohol and then from one-half part of ethyl acetate from which *p-cyanobenzenesulphonbutylamide* crystallized in flattened needles with a waxy texture, m.p. 99–100°. (Found: C, 55.5; H, 6.1; N, 11.9. $C_{11}H_{14}O_2N_2S$ requires C, 55.4; H, 5.9; N, 11.8 %.)

For amidine formation, the nitrile (3.37 g.) was suspended in absolute alcohol (5 c.c.) and converted into the imino-ether hydrochloride and then into the amidine in the usual way. The solid was collected and the ammoniacal mother liquor evaporated to dryness. The collected solid and the alcoholic residue were treated with water (15 c.c.) and extracted with ether to remove traces of unchanged nitrile. On concentration of the aqueous solution *p-sulphonbutylamidobenzamidinium hydrochloride* (2.63 g.), m.p. 127–128°, separated in hollow prismatic needles (negative crystals) and on recrystallization from an equal volume of water had m.p. 127–129°. (Found: C, 44.0; H, 6.5; N, 14.0; H_2O , 3.0. $C_{11}H_{17}O_2N_3S$, HCl, $\frac{1}{2}H_2O$ requires C, 43.9; H, 6.4; N, 13.6; H_2O , 3.0 %.)

p-Sulphondi-(β -hydroxyethyl)amidobenzamidinium hydrochloride. Technical diethanolamine (21.0 g.) in warm dry acetone (100 c.c.) was added fairly quickly to *p-cyanobenzenesulphonyl chloride* (23.35 g.) in acetone (100 c.c.). Reaction took place

immediately and was completed by digestion on the water-bath under reflux for 2 hr. The acetone was then distilled off, water added to the residue and the *p*-cyano-benzenesulphon-di-(β -hydroxyethyl)amide, yield 20.5 g., collected. It was crystallized from boiling ethyl alcohol (50 c.c.), finally from 12 vol. of boiling ethyl acetate and separated in elongated thin plates, m.p. 131–132°. (Found: C, 48.9; N, 5.2. $C_{11}H_{14}O_4N_2S$ requires C, 49.0; H, 5.5 %.)

For amidine formation, the above cyano-compound (9.0 g.) in ethyl alcohol (27 c.c.) was treated with dry hydrogen chloride and amidine formation completed as usual. The ammonium chloride which had separated was filtered off and the mother liquor boiled down, two crops (11.2 g. in all) being collected of crude amidine hydrochloride, which on crystallization from 95 % ethyl alcohol gave pure *p*-sulphon-di-(β -hydroxyethyl)amidobenzamidine hydrochloride (6.2 g.) crystallizing in thin plates, m.p. 203–204°. (Found: C, 40.9; H, 5.8. $C_{11}H_{17}O_4N_3S$, HCl requires C, 40.8; H, 5.6 %.)

p-Sulphonphenylamidobenzamidine hydrochloride. A solution of *p*-cyanobenzene-sulphonanilide (5.16 g.) (Remsen, Hartmann & Muckenfuss 1896) in absolute alcohol (10 c.c.) and dioxan (10 c.c.) was converted into the imino-ether and amidine formation completed as usual. The colourless, heavy, crystalline solid which separated from the alcoholic ammonia was collected (5.29 g.). The mother liquor gave a further quantity (1.42 g.) on evaporation. The combined solids were ground and washed with cold water (30 c.c.) and the residue suspended in water. 2*N*-Hydrochloric acid was carefully added to a permanent pH of about 6. The solution was filtered and evaporated to small bulk (about 4 c.c.), and the crystals (5.82 g.) which separated were collected. Recrystallization from a small volume of water afforded short stout prisms of *p*-sulphonphenylamidobenzamidine hydrochloride, m.p. 205–206°, with loss of water of crystallization below 120°. (Found: C, 47.7; H, 4.9; N, 13.5. Loss at 100° in a vacuum, 5.3. Found on dried material: C, 50.4; H, 4.6. $C_{13}H_{13}O_2N_3S$, HCl, H_2O requires C, 47.3; H, 4.9; N, 12.7; H_2O , 5.5. $C_{13}H_{13}O_2N_3S$, HCl requires C, 50.1; H, 4.5 %.)

4:4'-Guanylbzenesulphonamidobenzamidine dihydrochloride. *p*-Cyanobenzene-sulphonyl chloride (10 g.), *p*-cyanoaniline (5.9 g.) and dry pyridine (10 c.c.) were mixed and heated on the water-bath for 1.5 hr. The gum precipitated by water rapidly solidified; it was collected and dried, yield 13.4 g., m.p. 195°. Crystallization from aqueous methyl alcohol or, better, isopropyl alcohol, gave microscopic colourless prisms of 4:4'-dicyanobzenesulphonanilide, m.p. 200–201°. (Found: C, 59.6; H, 3.3. Calc. for $C_{14}H_9O_2N_3S$: C, 59.4; H, 3.2 %.) Ashley *et al.* (1942) record m.p. 201–202°.

A solution of the above dinitrile (4 g.) in absolute alcohol (8 c.c.) and dioxan (20 c.c.) was converted into the di-imino-ether hydrochloride and then into the diamidine in the usual manner. The hard crystalline deposit (3.9 g.) was collected and a further quantity (2.81 g.) obtained on evaporation of the alcoholic ammonia mother liquor to dryness. The solid, consisting of free di-amidine base (3.65 g.), was washed with a small volume of water and neutralized with 2*N*-hydrochloric acid. 4:4'-Guanylb-

benzenesulphonamidobenzamidine dihydrochloride hemihydrate was extremely soluble in water and separated from methyl alcohol-ethyl acetate (1:1) in clusters of colourless microscopic plates, m.p. 298–300° (efferv.). (Found: C, 42.1; H, 4.8. $C_{14}H_{15}O_2N_3S$, 2HCl, $\frac{1}{2}H_2O$ requires C, 42.1; H, 4.5 %.) Ashley *et al.* (1942) record m.p. 239° for a tetrahydrate.

p-Sulphonpiperidobenzamidine hydrochloride. Piperidine (10 c.c.) was added gradually to *p*-cyanobenzenesulphonyl chloride (11.7 g.), finely powdered and suspended in dry ether (100 c.c.) with external cooling. After 12 hr. the ether was distilled and the residue treated with water. The solid (13.1 g.) was collected and dissolved in the minimum volume (50 c.c.) of boiling ethyl alcohol. *p-Cyanobenzene-sulphonpiperidide*, so obtained, crystallized in pearly leaves, m.p. 134–135°. (Found: C, 57.4; H, 5.3; N, 11.2. $C_{12}H_{14}O_2N_2S$ requires C, 57.6; H, 5.6; N, 11.2 %.)

For amidine formation, the nitrile (5.26 g.) was suspended in absolute ethyl alcohol (15 c.c.) and the process completed as usual. The alcoholic ammonia was distilled to dryness, several intermediate crops of ammonium chloride being removed by filtration. The alcohol residue was dissolved in water, extracted with ether and then evaporated dry and fractionally crystallized from ethyl alcohol to separate ammonium chloride. Finally, crude *p-sulphonpiperidobenzamidine hydrochloride* (3.08 g.) separated and on crystallization from boiling ethyl alcohol (6 c.c.) afforded delicate hexagonal plates (2.05 g.), m.p. 224–225°. (Found, C, 47.5; H, 6.0. $C_{12}H_{17}O_2N_3S$, HCl requires C, 47.4; H, 6.0 %.)

p-Sulphonmorpholidobenzamidine hydrochloride. *p*-Cyanobenzenesulphonyl chloride (11.7 g.) suspended in dry ether (100 c.c.) was treated with morpholine (8.7 c.c.) with slight external cooling. After 24 hr. the ether was distilled, the residue diluted with water and the solid (13.56 g.) collected. *p-Cyanobenzenesulphon-morpholide* was crystallized from boiling ethyl alcohol (200 c.c.) and separated in pearly leaves, yield 11.5 g., m.p. 158–159°. (Found: C, 52.4; H, 4.9; N, 10.9. $C_{11}H_{13}O_3N_3S$ requires C, 52.4; H, 4.8; N, 11.1 %.)

For amidine formation, the nitrile (6.2 g.) was suspended in absolute ethyl alcohol (18.6 c.c.) and the process carried through as usual. The clear alcoholic ammonia solution was evaporated to dryness, several crops of ammonium chloride being removed during the process. The residue was dissolved in water, made slightly acid with hydrochloric acid, and unchanged nitrile removed by ether. On concentration to a small volume, the aqueous solution gave *p-sulphonmorpholidobenzamidine hydrochloride* (3.39 g.), m.p. 100°. If dried at 110° it melts to a clear liquid at 235° with previous sintering. It was best crystallized from 5 vol. of isopropyl alcohol, the hydrated crystals becoming white and opaque before solution was effected. This salt separated in clusters of needles or prisms, m.p. 235–236°, which still retained one molecule of water of crystallization, not lost at 100°. (Found: C, 40.8; H, 5.8; N, 12.8. $C_{11}H_{15}O_3N_3S$, HCl. H_2O requires C, 40.8; H, 5.6; N, 13.0 %.)

p-Guanylbzenesulphonamidouacetamidine dihydrochloride. Aminoacetonitrile sulphate (5.25 g.) was suspended in pyridine (15 c.c.) and *p*-cyanobenzenesulphonyl chloride (10.5 g.) added as a fine powder, the reaction being controlled by external

cooling. After keeping at 37° for 12 hr., the clear solution was poured into excess 3N-hydrochloric acid, the solid collected, washed with water, ground with sodium bicarbonate solution and again collected and well washed. *p*-Cyanobenzenesulphonamidoacetonitrile (6.04 g.) separated from water (150 c.c.) in leaflets, m.p. 139–140°. (Found: C, 48.5; H, 3.1. $C_9H_7O_2N_3S$ requires C, 48.8; H, 3.2 %.)

For di-amidine formation, the di-nitrile (5.7 g.) was dissolved in a mixture of absolute ethyl alcohol (20 c.c.) and dioxan (10 c.c.) with the aid of heat. Di-imino-ether formation and finally di-amidine formation were effected in the usual manner. The mixture of di-amidine base and ammonium chloride which had separated at 37° was treated with a little water, when it dissolved and a crystalline solid rapidly separated. This was collected, dissolved in N-hydrochloric acid (10 c.c.) and the solution concentrated to a small volume. *p*-Guanylbzenesulphonamidoacetamidine dihydrochloride separated in tablets, yield 3.3 g. It was recrystallized from water (2.3 c.c.) and gave the pure salt, m.p. 204°. (Found: C, 29.1; H, 5.5; N, 19.0; H_2O , 11.7. $C_9H_{13}O_2N_5S \cdot 2HCl \cdot 2\frac{1}{2}H_2O$ requires C, 28.9; H, 5.4; N, 18.8; H_2O , 12.1 %.)

p-Guanylbzenesulphonylguanidine hydrochloride (XVII). *p*-Cyanobenzenesulphonyl chloride (32 g.), *S*-methylisothiouraea sulphate (22.25 g.) and dry pyridine (60 c.c.) were mixed and warmed gently on the water-bath. A vigorous reaction took place and rapidly subsided. After an hour the solution was added to water, when the precipitated gum slowly crystallized. *p*-Cyanobenzenesulphonyl-*S*-methylisothiouraea (14.5 g.) crystallized from water in colourless flat prisms, m.p. 132–133°. (Found: C, 42.3; H, 3.3; N, 16.3; S, 25.6. $C_9H_9O_2N_3S_2$ requires C, 42.3; H, 3.5; N, 16.5; S, 25.1 %.)

A solution of the above thiouraea (5.1 g.) in absolute alcohol (5 c.c.) and dioxan (25 c.c.) was converted into the imino-ether hydrochloride in the usual way. The residue, after removal of solvent and excess hydrogen chloride, was treated with 10 % alcoholic ammonia (100 c.c.) for 7 days. The resulting solution was freed from methyl mercaptan, excess ammonia and solvent, the residual gum crystallizing on boiling with a small volume of alcohol. The product, *p*-guanylbzenesulphonylguanidine hydrochloride, was crystallized from methyl alcohol and then from water (4.8 g., in 3 c.c.), when clusters of colourless radiating prisms (3.9 g.) separated, m.p. 165–170° on slow heating or as low as 140° in a preheated bath. (Found: on solid dried over H_2SO_4 in a vacuum, N, 23.6; H_2O , 5.4. $C_9H_{11}O_2N_5S \cdot HCl \cdot H_2O$ requires N, 23.7; H_2O , 6.1. Found, on solid dried at 110° in a vacuum, C, 34.2; H, 4.6. $C_9H_{11}O_2N_5S \cdot HCl$ requires C, 34.6; H, 4.3 %.)

p-Guanylbzenesulphonamidothiazole hydrochloride. 2-Aminothiazole (10.0 g.), *p*-cyanobenzenesulphonyl chloride (10.1 g.) and dry ether (110 c.c.) were mixed at 0° and kept at room temperature overnight. The heterogeneous mixture was gently digested on the water-bath to complete the reaction and then freed from ether by distillation. Water was then added and the undissolved solid collected, yield 11.13 g. This was treated with 2N-sodium hydroxide solution (20 c.c.) and the non-acidic material (1.73 g.) collected. The alkaline filtrate was acidified with acetic acid and the solid (6.9 g.) collected. The alkali-insoluble solid, on boiling with ethyl alcohol

(15 c.c.) insufficient to dissolve it, gave on cooling a solid (1.6 g.), m.p. 218°. This was then crystallized from 53 vol. of boiling glacial acetic acid and gave 2-*p*-cyanobenzenesulphonimido-3-*p*-cyanobenzenesulphonylthiazole, in compact tablets, m.p. 226° (decomp.). (Found: C, 47.3; 47.5; H, 2.8; 2.9; N, 12.7; 13.1. $C_{17}H_{10}O_4N_4S_2$ requires C, 47.4; H, 2.3; N, 13.0 %.) The alkali-soluble portion when boiled with ethyl alcohol (25 c.c.), insufficient to dissolve it, gave, on cooling, a solid (6.15 g.), m.p. 208°. It was crystallized first from 7 vol. of boiling glacial acetic acid and separated in blades, m.p. 214–215° raised to 215–216° by further crystallization from 40 vol. of ethyl alcohol. Analysis showed it to be the required 2-*p*-cyanobenzenesulphonamidothiazole. (Found: C, 45.2; H, 2.7; N, 15.8. $C_{10}H_7O_2N_2S_2$ requires C, 45.3; H, 2.7; N, 15.8 %.)

This latter substance was more readily obtained by working in a homogeneous pyridine solution. Thus, 2-aminothiazole (5.0 g.) in dry pyridine (20 c.c.) was treated with *p*-cyanobenzenesulphonyl chloride (11.7 g.) and the solution warmed on the water-bath for 1 hr. The cooled solution, poured into excess 3*N*-hydrochloric acid, gave a solid which was almost completely soluble in 2*N*-sodium hydroxide solution (25 c.c.). On acidification with glacial acetic acid, the crude cyano-compound (12.3 g.), m.p. 207°, was obtained. It was recrystallized from glacial acetic acid (75 c.c.) and separated in glistening leaflets (10.5 g.), m.p. 214°.

This cyano-compound (5.3 g.) was suspended in absolute ethyl alcohol (15 c.c.) and dioxan (15 c.c.), and imino-ether formation and subsequently amidine formation effected in the usual way. The alcoholic ammonia solution, after keeping at 37°, had deposited a brown solid which was collected and, on evaporation of the mother liquor, a succession of crops of crystalline material was obtained. These were all combined and treated with a small volume of water (15 c.c.) which removed ammonium chloride and a small amount of amidine hydrochloride. The insoluble material (7.4 g.) was extracted with dilute hydrochloric acid which removed the amidine and left unchanged nitrile (2.3 g.), m.p. 214°. The above aqueous extract, and the hydrochloric acid extract gave, on concentration, *p*-guanylbenzenesulphonamidothiazole hydrochloride (4.2 g.), which was recrystallized from 3 parts of hot water, giving the pure salt in the form of thin rhomb-shaped leaflets, m.p. 283–284°. (Found: C, 37.5; H, 3.7; N, 17.5. $C_{10}H_{10}O_2N_4S$, HCl requires C, 37.7; H, 3.5; N, 17.6 %.)

2-*p*-Guanylbenzenesulphonamido-4-methylthiazole hydrochloride. *p*-Cyanobenzenesulphonyl chloride (10 g.), 2-amino-4-methylthiazole (6 g.) and dry pyridine (10 c.c.) were warmed on the water-bath for 1½ hr. The product 2-*p*-cyanobenzenesulphonamido-4-methylthiazole (12.8 g.) isolated in the usual way separated from 50 % aqueous alcohol in prisms which tenaciously retained a light buff colour, m.p. 184–185°. (Found: C, 47.5; H, 3.5; N, 15.0. $C_{11}H_9O_2N_2S_2$ requires C, 47.3; H, 3.2; N, 15.1 %.)

The preceding nitrile (7.4 g.) was treated with hydrogen chloride in absolute alcohol (15 c.c.) and dioxan (30 c.c.) and amidine formation effected in the customary manner. The yellow solid which separated was collected (7.45 g.). It was suspended

in water and 2*N*-hydrochloric acid carefully added, but it was not possible to dissolve the solid entirely above pH 4. Insoluble material was filtered off and the colourless solution gave a further bright yellow precipitate when the pH was raised to about 5.5. The colourless filtrate was evaporated to small bulk and the crystals of 2-*p*-guanylbenezesulphonamido-4-methylthiazole hydrochloride (1.33 g.) which separated were collected. Recrystallization from 2.5 parts of water afforded colourless rectangular plates which effloresced in a vacuum over concentrated sulphuric acid, m.p. 220–221° (decomp.). (Found: Loss at 100° in a vacuum, 5.3. On dried material, C, 39.6; H, 4.3. $C_{11}H_{12}O_2N_4S_2$, HCl, H_2O requires H_2O , 5.1. $C_{11}H_{12}O_2N_4S_2$, HCl requires C, 39.7; H, 3.9 %.)

2'-*p*-Guanylbenezesulphonamido-4':6'-dimethylpyrimidine hydrochloride. 2-Sulphanilamido-4:6-dimethylpyrimidine ('Sulphamezathine') (13.9 g.) was diazotized in hydrochloric acid solution (H_2O , 36 c.c.; HCl, 32 %, 11.6 c.c.) and the diazo-solution added at 0° to a vigorously stirred solution of sodium cyanide (4.9 g.) and cuprous cyanide (4.49 g.) in water (40 c.c.). When all had been added the solution was warmed to 60° for 1 hr., then cooled and the crude solid collected and dried. It was extracted with ether (Soxhlet) and gave crude *p*-cyanobenezesulphonamido-dimethylpyrimidine (6.3 g., m.p. 192°). The aqueous liquors on ether extraction gave a further crop (0.7 g.), m.p. 192°. It was soluble in 10 parts of boiling spirit and separated in pale yellow thin prisms, m.p. 196–197°. (Found: C, 54.3; H, 4.5. $C_{13}H_{12}O_2N_4S$ requires C, 54.1; H, 4.2 %.)

The above nitrile (8.83 g.) was dissolved in dioxan (16 c.c.), and absolute ethyl alcohol (24 c.c.) and imino-ether followed by amidine formation were effected as usual. The copious crystalline deposit which separated from the ammoniacal alcoholic solution proved to be crude amidine base (10.0 g.). It was treated with *N*-hydrochloric acid (30 c.c.) and the solution on concentration gave *p*-guanylbenezesulphonamidodimethylpyrimidine hydrochloride (9.2 g.). It was recrystallized from an equal weight of water and separated in two forms, tablets and spiked plates. These have no sharp m.p.; they shrink considerably between 200 and 205°, do not form a meniscus, and effervesce about 230°. (Found: C, 43.0; H, 4.9; N, 19.7; H_2O , 5.6. $C_{13}H_{16}O_2N_5S$, HCl, H_2O requires C, 43.4; H, 5.0; N, 19.5; H_2O , 5.0 %.)

p-Sulphon-2-pyridylamidobenzamidine hydrochloride. *p*-Cyanobenezesulphonyl chloride (10 g.), 2-aminopyridine (5 g.) and dry pyridine (10 c.c.) were mixed and warmed on the water-bath for 1½ hr. The resulting viscous mass was shaken with water and the crystalline product (11.9 g.) collected and dried. Crystallization from spirit afforded colourless, pearly plates of 2-*p*-cyanobenezesulphonamidopyridine, m.p. 193–194°. (Found: C, 55.4; H, 3.6; N, 15.6. $C_{12}H_8O_2N_5S$ requires C, 55.6; H, 3.5; N, 16.2 %.)

The foregoing nitrile (4 g.) in absolute alcohol (12 c.c.) and dioxan (40 c.c.) was converted into the amidine through the imino-ether hydrochloride in the usual way. The microcrystalline white solid (4.74 g.) which separated was collected and a further quantity (1.1 g.) obtained by evaporating the mother liquor to dryness. The substance was neutralized with 2*N*-hydrochloric acid to about pH 6 and the filtered solution

evaporated to small bulk, when crystallization occurred on cooling. Recrystallization from a small volume of water afforded small short rhombs of *p*-sulphon-2-pyridylamidobenzamidine hydrochloride, m.p. 207° (decomp.). (Found: C, 46.2; H, 4.2; N, 17.6. $C_{12}H_{12}O_2N_4S$, HCl requires C, 46.1; H, 4.2; N, 17.9 %.)

p-Sulphon-*O*-methylhydroxylamidobenzamidine hydrochloride. For the preparation of this amidine, *O*-methylhydroxylamine base was required. For this purpose dipotassium hydroxylaminedisulphonate was prepared as described by Rollefson & Oldershaw (1932). This salt is unstable and rapidly develops free sulphate, so it must be methylated whilst still slightly damp. About 105 g. of this salt (cf. Traube, Ohlendorf & Zander 1920) were added to a solution of potassium hydroxide (28 g.) in water (250 c.c.) at room temperature and dimethyl sulphate (35 c.c.), freshly distilled and acid-free, was run in from a dropping-funnel. The solid in suspension slowly dissolved and the temperature rose during the reaction to about 35°. The alkalinity of the final solution having been reduced by a stream of carbon dioxide, the solution on concentration under reduced pressure below 50° gave dipotassium *O*-methylhydroxylaminedisulphonate in the form of bold hexagonal plates in good yield. This salt (60.2 g.) was boiled with hydrochloric acid (100 c.c., 16 %) for 5 hr., the solution made strongly alkaline with sodium hydroxide and the liberated *O*-methylhydroxylamine steam distilled into *N*-hydrochloric acid (100 c.c.). The latter on evaporation to dryness gave *O*-methylhydroxylamine hydrochloride which was collected after dissolving in a little hot ethyl alcohol and adding excess of hot ethyl acetate, yield 15.7 g. To obtain anhydrous base, the hydrochloride (32.6 g.) was treated dropwise with 50 % sodium hydroxide solution and the issuing basic vapour passed over pellets of potash in a U-tube kept at 60°. The *O*-methylhydroxylamine base was collected in a freezing mixture, yield 90 %.

O-Methylhydroxylamine (6.78 g.) was dissolved in dry ether (25 c.c.) and *p*-cyanobenzenesulphonyl chloride (6.78 g.) added all at once. The reaction commenced immediately and was completed by boiling on the water-bath for a short time. Water was then added and then the ether was distilled off, leaving a crystalline solid, yield 7.0 g. *p*-Cyanobenzenesulphon-*O*-methylhydroxylamide so obtained was crystallized from 17 parts of boiling benzene and separated in tablets, m.p. 118–119°. (Found: C, 45.2; H, 3.8; N, 13.3. $C_8H_8O_3N_2S$ requires C, 45.2; H, 3.8; N, 13.2 %.)

For amidine formation the above nitrile (4.24 g.) was dissolved in absolute alcohol (12 c.c.) and the process completed in the usual way. The alcoholic ammonia solution deposited a crystalline solid (4.4 g.), which was chloride-free, and on crystallization from ethyl alcohol (50 mg. in 30 c.c.), in which solvent it is sparingly soluble, gave *p*-sulphon-*O*-methylhydroxylamidobenzamidine base (30 mg.), m.p. 214° (with vigorous efferv.). (Found: C, 41.8; H, 5.0; N, 18.3. $C_8H_{11}O_3N_3S$ requires C, 41.9; H, 4.8; N, 18.3 %.) The main bulk of base was treated with *N*-hydrochloric acid (19.0 c.c.) and the solution concentrated to a small volume. *p*-Sulphon-*O*-methylhydroxylamidobenzamidine hydrochloride (4.24 g.) separated in large tablets, m.p. 235–236° (efferv.). (Found: C, 36.3; H, 4.7; N, 15.9. $C_8H_{11}O_3N_3S$, HCl requires C, 36.1; H, 4.6; N, 15.8 %.)

p-Sulphon-*O*-ethylhydroxylamidobenzamidine hydrochloride. This substance involves the initial preparation of *O*-ethylhydroxylamine. Hydroxyurethane (35 g.) (Hantzsch 1894) was added to a cold solution of sodium hydroxide (13.33 g.) in ethyl alcohol (130 c.c.) followed by ethyl iodide (52 g.). The mixture was boiled for 6 hr., the alcohol removed and the crude *O*-ethylhydroxyurethane extracted with ether (yield 26.8 g.). This was hydrolysed by boiling for 2 hr. with a solution of sodium hydroxide (32 g.) in water (32 c.c.), the *O*-ethylhydroxylamine formed being extracted with ether and converted into the hydrochloride (12.75 g.) by evaporation with excess aqueous hydrochloric acid. Anhydrous *O*-ethylhydroxylamine was recovered from the hydrochloride by the method described above for the *O*-methylhydroxylamine, the yield being 39 % on the crude hydroxyurethane used as starting material.

p-Cyanobenzenesulphon-*O*-ethylhydroxylamide was prepared by dissolving ethylhydroxylamine (2.65 g., 4 mol.) in dry ether (10 c.c.), and adding *p*-cyanobenzenesulphonyl chloride (2.0 g., 1 mol.) all at once. The reaction was completed by warming on the water-bath for an hour. The solvent was removed, water added and the hydroxylamide (1.84 g., yield 84 %) collected. It separated from its own weight of benzene in prisms, m.p. 98–99°. (Found: C, 47.9; H, 4.4; N, 12.4. $C_9H_{10}O_3N_2S$ requires C, 47.8; H, 4.5; N, 12.4 %.) If molecular proportions of base and acid chloride were mixed in pyridine, the yield of the amide was much reduced.

For amidine formation the nitrile (6.3 g.) in ethyl alcohol (18 c.c.) was converted into the imino-ether hydrochloride and then into the amidine as usual. The alcoholic ammonia solution kept at 37° deposited large prisms (6.0 g.) free from ammonium chloride. A portion (50 mg.) was dissolved in the minimum volume of boiling absolute alcohol (24 c.c.) and separated in soft needles, m.p. 222° (efferv.), which proved on analysis to be *p*-sulphon-*O*-ethylhydroxylamidobenzamidine base. (Found: C, 44.4; H, 5.7; N, 17.1. $C_9H_{13}O_3N_3S$ requires C, 44.4; H, 5.4; N, 17.3 %.) The main bulk of base (6.0 g.) was treated with the calculated amount of *N*-hydrochloric acid (24.7 c.c.) and the solution concentrated. The hydrochloride crystallized in large rhombs, yield 6.63 g., m.p. 222° (efferv.). (Found: C, 37.6; H, 5.4; N, 14.4; H_2O , 3.1. $C_9H_{13}O_3N_3S$, HCl, $\frac{1}{2}H_2O$ requires C, 37.4; H, 5.2; N, 14.5; H_2O , 3.1 %.)

p-Sulphon-*O*-propylhydroxylamidobenzamidine hydrochloride. *O*-Propylhydroxylamine was prepared by hydrolysis of *O*-propylhydroxyurethane (see preparation of *O*-ethyl analogue above) with strong potash (34 g. KOH in 68 c.c. H_2O). The base was collected in ether, thoroughly dried over potassium hydroxide and then fractionally distilled. From crude hydroxyurethane a fraction, b.p. 82–84° at 754 mm., was collected, yield 85 %. A portion was converted into *O*-propylhydroxylamine hydrochloride, which is best crystallized by solution in a little hot ethyl alcohol followed by addition of excess hot ethyl acetate. It crystallizes in pearly scales, m.p. 154–155°. (Found: C, 32.4; H, 8.8. C_8H_9ON , HCl requires C, 32.3; H, 9.0 %.)

p-Cyanobenzenesulphon-*O*-propylhydroxylamide was obtained by adding *p*-cyanobenzenesulphonyl chloride (10.05 g.) in portions to *O*-propylhydroxylamine (3.75 g.) in dry pyridine (10 c.c.) with external cooling. The solution was kept at 37° for 24 hr.,

then poured into 3*N*-hydrochloric acid (50 c.c.) and the solid collected, washed, ground with sodium bicarbonate solution, again collected and washed with water, yield 7.5 g. It separated from boiling ethyl alcohol in needles, m.p. 135–136°. (Found: C, 50.0; H, 5.1; N, 11.5. $C_{10}H_{12}O_3N_3S$ requires C, 50.0; H, 5.0; N, 11.7 %.) The yield by this method is poor and could probably be improved by use of excess of base in ether and avoiding pyridine as described for the *O*-ethyl analogue above.

The nitrile (4.8 g.) was dissolved in ethyl alcohol (9.6 c.c.) and dioxan (4.8 c.c.) and the process of amidine formation carried through as usual. The yellow crystalline solid (4.4 g.) which separated from the ammoniacal alcoholic solution was chloride-free. It was very sparingly soluble in boiling ethyl alcohol, more soluble in methyl alcohol, one part requiring 200 parts of boiling solvent for solution. *p*-Sulphon-*O*-propylhydroxylamidobenzamidine separated from this solvent in silky primrose-yellow needles, m.p. 222–223° (efferv.). (Found: C, 46.6; H, 5.9; N, 16.1. $C_{10}H_{15}O_3N_3S$ requires C, 46.7; H, 5.9; N, 16.3 %.) The main bulk of the base (4.3 g.) was treated with *N*-hydrochloric acid (16.5 c.c.) and the solution evaporated to a small volume. The *hydrochloride* separated as clusters of prisms, m.p. 188–189°, yield 4.2 g. (Found: C, 40.7; H, 5.5. $C_{10}H_{15}O_3N_3S$, HCl requires C, 40.9; N, 5.5 %.)

p-Sulphon-*O*-butylhydroxylamidobenzamidine *hydrochloride*. *O*-Butylhydroxylamine hydrochloride (8.75 g.), prepared from *O*-butylhydroxyurethane, was dissolved in dry pyridine (21 c.c.) and treated with *p*-cyanobenzenesulphonyl chloride (14.07 g.). After keeping for 24 hr. at 37°, the solution was diluted with 3*N*-hydrochloric acid and the solid (18.4 g.) collected. It was treated with excess of *N*-sodium hydroxide solution which dissolved *p*-cyanobenzenesulphon-*O*-butylhydroxylamide and left *di*-*p*-cyanobenzenesulphon-*O*-butylhydroxylamide undissolved. The former (11.8 g.) was recovered by acidification and on crystallization from methyl alcohol (10 c.c.) separated in needles, yield 10.3 g., m.p. 121°. (Found: C, 52.0; H, 5.5; N, 10.9. $C_{11}H_{14}O_3N_2S$ requires C, 51.9; H, 5.5; N, 11.0 %.) The latter (3.2 g.) separated from glacial acetic acid (40 c.c.) in microscopic tablets, m.p. 205°. (Found: C, 51.6; H, 4.2; N, 9.9. $C_{18}H_{17}O_5N_3S_2$ requires C, 51.5; H, 4.1; N, 10.0 %.)

For amidine formation, the mono-cyano compound (5 g.) was dissolved in absolute ethyl alcohol (15 c.c.) and worked through as usual. Well-formed prisms (4.43 g.) separated from the alcoholic ammonia solution and they were chloride-free. A portion (0.1 g.) was dissolved in boiling absolute alcohol (20 c.c.) and on cooling *p*-sulphon-*O*-butylhydroxylamidobenzamidine base separated in small needles, m.p. 218° (efferv.). (Found: C, 48.5; H, 6.4; N, 15.4. $C_{11}H_{17}O_3N_3S$ requires C, 48.7; H, 6.3; N, 15.5 %.) The remainder of the base (4.3 g.) was dissolved in *N*-hydrochloric acid (15.9 c.c.), and on concentration the solution deposited the *hydrochloride* (4.73 g.) in tablets or short prisms, m.p. (air-dry) 110–114°; dried at 95° it forms a meniscus about 135–137°. (Found: C, 42.1; H, 6.1; N, 13.6; H_2O , 2.9. $C_{11}H_{17}O_3N_3S$, HCl, $\frac{1}{2}H_2O$ requires C, 41.7; H, 6.0; N, 13.3; H_2O , 2.8 %.)

p-2':5'-Dihydroxyphenylsulphonylbenzamidine *hydrochloride*. *p*-Cyanobenzenesulphinic acid (4.2 g.) was dissolved in warm water (20 c.c.) and added to an aqueous solution (40 c.c.) of *p*-quinone (2.7 g.). The colour of quinone was immediately dis-

charged, and after warming the mixture for a few minutes on the water-bath it was cooled and the product collected in quantitative yield. Crystallization from 10 % aqueous alcohol gave fine prisms of 2-p'-cyanophenylsulphonylhydroquinone with a slight yellow tinge, m.p. 182°. (Found: C, 56.5; H, 3.4. $C_{13}H_9O_4NS$ requires C, 56.7; H, 3.3 %.)

A solution of the above nitrile (7 g.) in absolute alcohol (7 c.c.) and dioxan (10 c.c.) was converted into the amidine in the usual manner. The bright yellow solid which separated was collected and dried (8.6 g.); only traces were obtained on evaporating the alcoholic mother liquor to dryness. The solid consisting of free amidine base was suspended in water and 2N-hydrochloric acid added until the substance just dissolved. The solution was concentrated to small bulk (about 5 c.c.) and on cooling deposited microscopic prisms. *Dihydroxyphenylsulphonylbenzamidine hydrochloride* was recrystallized from a small volume of water and had m.p. 248° (decomp.) after softening at 220°. (Found: Loss at 100° in a vacuum, 4.1. $C_{13}H_{12}O_4N_2S$, HCl, H_2O requires H_2O , 5.2 %. On dried material, C, 47.3; H, 4.1; N, 8.1. $C_{13}H_{12}O_4N_2S$, HCl requires C, 47.5; H, 4.0; N, 8.5 %.)

p-Methylsulphonylbenzamidine hydrochloride (XX). Methyl *p*-aminophenylsulphone (15.4 g.) was submitted to the Sandmeyer reaction under the conditions described above for sulphanilamide; *p-methylsulphonylbenzonitrile* (9.0 g.) separated from water in fine colourless needles, m.p. 141°. (Found: N, 7.7. $C_8H_7O_2NS$ requires N, 7.7 %.)

The nitrile (7.2 g.) finely powdered was added to alcohol (6 c.c.) and dioxan (12 c.c.) and the stages of amidine formation followed in the usual way. The heavy solid (7.84 g.) which separated from the ammoniacal alcohol was collected and the mother liquor on evaporation gave a further quantity (3.3 g.). *p-Methylsulphonylbenzamidine hydrochloride* separated from methyl alcohol in colourless rhombic prisms, m.p. 288–289°, with slight softening about 265°. (Found: N, 11.9; Cl, 15.1. $C_8H_{10}O_2N_2S$, HCl requires N, 11.9; Cl, 15.1 %.)

Sulphonamidobenzamidines substituted in the amidine group

p-Sulphonamidobenzmethylamidine and *p-sulphonamidobenz-NN'-dimethylamidine hydrochlorides*. *p*-Cyanobenzenesulphonamide (4.55 g.) in absolute alcohol (12 c.c.) was converted into the imino-ether hydrochloride, which, on removal of the solvent and as much hydrogen chloride as possible at the pump, was added to ethyl alcoholic methylamine solution (25 c.c., 33 %) and kept at 37° for 3 weeks. The thick mass of crystals which separated was collected, yield 5.07 g. It was chloride-free and on crystallization from water (0.1 g. in 3 c.c.) gave glistening leaflets of *p-sulphonamidobenzmethylamidine* base, m.p. 222° (efferv.). (Found: C, 44.8; H, 5.2; N, 19.5. $C_8H_{11}O_2N_3S$ requires C, 45.0; H, 5.2; N, 19.7 %.) The main bulk of solid was treated with N-hydrochloric acid (21 c.c.) and the solution on concentration deposited the *hydrochloride* in prisms (3.87 g.), m.p. 253–254°. (Found: C, 38.6; H, 4.7; N, 17.1. $C_8H_{11}O_2N_3S$, HCl requires C, 38.5; H, 4.8; N, 16.8 %.) The original ammoniacal

alcohol mother liquor was concentrated and gave a crop of crystals which readily deliquesced in part leaving plates (0.45 g.) which, crystallized from water (0.75 c.c.), deposited tablets, m.p. 310° , of *p*-sulphonamidobenz-*NN'*-dimethylamidine hydrochloride. (Found: C, 40.9; H, 5.5; N, 16.0. $C_9H_{13}O_3N_3S$, HCl requires C, 41.0; H, 5.4; N, 15.9 %.) The final alcoholic mother liquor on concentration to a small volume gave nodules (50 mg.) which on crystallization from water (0.5 c.c.) gave leaflets, m.p. $208-209^{\circ}$, of *p*-sulphonamidobenzmethylamide. (Found: C, 44.7; H, 4.9; N, 12.8. $C_8H_{10}O_3N_3S$ requires C, 44.8; H, 4.7; N, 13.1 %.)

When the reaction of the imino-ether hydrochloride and alcoholic methylamine was carried out at 50° instead of 37° there was a slightly increased yield of the dimethylamidine.

p-Sulphondimethylamidobenz-*N*-methylamidine picrate. *p*-Sulphondimethylamidobenzoic acid (34.4 g.) prepared in a manner similar to that described by de Jong (1923) was refluxed with thionyl chloride (50 c.c.) for 3.5 hr. Excess thionyl chloride was recovered and the last traces were entrained by distillation with two lots of dry benzene. The solid chloride was finely powdered, kept in a vacuum for an hour and then added in portions with cooling to excess of 30 % methylamine solution (50 c.c.). The mixture was shaken vigorously and the product collected after 2 hr. Crystallization from water afforded *p*-sulphondimethylamidobenzmethylamide, colourless plates (28.8 g.), m.p. 156° . (Found: C, 49.8; H, 5.9. $C_{10}H_{14}O_3N_3S$ requires C, 49.6; H, 5.8 %.)

The preceding amide (6.05 g.) was suspended in toluene (75 c.c.) of which part (15 c.c.) was distilled away to ensure dryness, and powdered phosphorus pentachloride (5.7 g., 10 % excess) was added. The mixture was warmed on the water-bath for an hour and then toluene and phosphoryl chloride were thoroughly removed in a vacuum and the crystalline residue treated with 10 % alcoholic ammonia solution at 37° . The solution gave on evaporation a syrupy hydrochloride which could not be crystallized. *p*-Sulphondimethylamidobenz-*N*-methylamidine picrate, obtained by addition of excess sodium picrate solution, separated from 80 % aqueous alcohol in orange plates, m.p. 222° (softening at 220°); on two occasions recrystallization gave crystals (polymorphic form?) of m.p. 260° , but this material was not regularly obtainable. (Found: C, 40.4; H, 3.5; N, 18.4. $C_{10}H_{16}O_4N_3S$, $C_6H_5O_2N_3$ requires C, 40.8; H, 3.8; N, 17.9 %.) The picrate was recovered unchanged after treatment with seven molecular proportions of alcoholic hydrochloric acid.

p-Sulphondimethylamidobenz-*NN'*-dimethylamidine hydrochloride. *p*-Sulphondimethylamidobenzmethylamide (6.05 g.) was treated with phosphorus pentachloride as described above and the imino-chloride treated with 33 % alcoholic methylamine solution (25 g.) at 37° . On solution the product was contaminated with some of the starting material, which was removed by extracting an aqueous solution with chloroform. The required hydrochloride (5.7 g.) separated from water in well-developed, colourless prisms, m.p. $296-298^{\circ}$. (Found: C, 45.6; H, 6.3; N, 14.3. $C_{11}H_{17}O_4N_3S$, HCl requires C, 45.3; H, 6.2; N, 14.4 %.)

p-Sulphondimethylamidobenz-NNN'-trimethylamidine picrate. *p*-Sulphondimethylamidobenzmethylamide was treated with (i) phosphorus pentachloride, and (ii) alcoholic dimethylamine solution, but the resulting syrupy amidine hydrochloride could not be obtained in a crystalline condition. The *picrate* separated from 20 % aqueous methyl alcohol in pale yellow prisms, m.p. 182–183°. (Found: C, 43.4; H, 5.0; N, 16.7. $C_{12}H_{19}O_2N_3S$, $C_6H_3O_7N_3$ requires C, 43.4; H, 4.4; N, 16.9 %.)

p-Sulphonamidobenzamidrazone hydrochloride (VI). *p*-Cyanobenzenesulphonamide (4.5 g.) was converted into the imino-ether hydrochloride, which, as a dry powder, was suspended in absolute ethyl alcohol (10.0 c.c.) and treated with hydrazine hydrate (5.0 c.c.). A clear solution resulted, but was rapidly followed by crystallization. The collected solid (5.38 g.) was treated with *N*-hydrochloric acid (11 c.c.) and, on concentration of the solution, a very soluble salt, *p-sulphonamidobenzamidrazone hydrochloride*, separated in aggregates of thin plates (4.53 g.). On crystallization from water (1.5 c.c.) it had m.p. 187° (efferv.). (Found: C, 33.8; H, 4.0; N, 22.0. $C_7H_{10}O_2N_4S$, HCl requires C, 33.5; H, 4.4; N, 22.4 %.)

2'-p-Sulphonamidophenyl-4':8'-dimethylpyrimidine (VII). *p*-Sulphonamidobenzamidine base (2.13 g.) and acetylacetone (1 c.c.) were heated for 24 hr. at 125–130° (external bath), this length of time being necessary to effect complete solution. The reaction mixture was cooled, mixed with a little ethyl alcohol and the crystalline *sulphonamidophenyldimethylpyrimidine* collected, yield 1.8 g. It separated from 10 parts of boiling alcohol in needles, m.p. 193–194°. (Found: C, 51.6; H, 5.4; N, 15.2; H_2O , 6.5. $C_{12}H_{13}O_2N_3S$, H_2O requires C, 51.2; H, 5.4; N, 15.0; H_2O , 6.5 %.) A repetition of this preparation on a slightly larger scale gave *p-sulphonamidobenzamide* as a by-product, m.p. 242° not depressed by admixture with an authentic specimen. (Found: C, 42.3; H, 4.1. Calc. for $C_7H_8O_2N_2S$: C, 42.0; H, 4.0 %.)

Amidines and amidoximes of double sulphonamides

p-Sulphon-(p'-sulphamylphenyl)amidobenzamidine nitrate. Sulphanilamide (8.6 g.) dissolved in dry pyridine (20 c.c.) was treated with *p*-cyanobenzenesulphonyl chloride (11.7 g.) and the solution heated for 1 hr. on the boiling water-bath. On adding excess of 3*N*-hydrochloric acid, a solid was precipitated, which was collected, washed and dried and crystallized from the minimum volume of boiling glacial acetic acid (200 c.c.). *p-Cyanobenzenesulphonamidobenzenesulphonamide* (15.1 g.) separated on cooling in clusters of fine needles, m.p. 221–222°. (Found: C, 46.4; H, 3.6. $C_{13}H_{11}O_4N_3S_2$ requires C, 46.3; H, 3.3 %.)

For amidine formation, the above nitrile (8.4 g.) was dissolved in dioxan (25 c.c.) and ethyl alcohol (17 c.c.) and the process continued as usual. The alcoholic ammonia solution was separated from ammonium chloride and concentrated, when crude amidine base (6.89 g.) separated. This was washed with water and neutralized with 2*N*-hydrochloric acid (6.55 c.c.). All attempts to crystallize the amidine hydrochloride were unsuccessful, but on adding ammonium nitrate to the aqueous solution *p-sulphon-(p'-sulphamylphenyl)amidobenzamidine nitrate* (5.89 g.) readily separ-

ated. It was then crystallized from isopropyl alcohol (50 c.c.) with addition of water (3 c.c.) and separated in aggregates of diamond-shaped plates, m.p. 208–209° (efferv.). (Found: C, 37.3; H, 4.0; N, 17.0. $C_{13}H_{14}O_4N_4S_2$, HNO_3 requires C, 37.4; H, 3.6; N, 16.8 %.)

p-Sulphon-(p'-NN'-dimethylsulphamylphenyl)amidobenzamidine hydrochloride. *p*-Cyanobenzenesulphonyl chloride (15.1 g.) was added to a solution of *N*¹-dimethylsulphanilamide (15 g.) in pyridine (30 c.c.). After 12 hr. excess of 3*N*-hydrochloric acid was added and the product collected and well washed with water, yield 26 g. It was dissolved in the minimum volume of boiling glacial acetic acid (130 c.c.) and *p*-cyanobenzenesulphonamidobenzenesulphondimethylamide, m.p. 206–207°, separated in plates. (Found: C, 48.9; H, 4.2; N, 11.7. $C_{15}H_{15}O_4N_3S_2$ requires C, 49.3; H, 4.1; N, 11.7 %.)

For amidine formation, the nitrile (9.1 g.) was dissolved in a mixture of absolute alcohol (30 c.c.) and dioxan (30 c.c.) and the process carried through in the usual way. The crystalline deposit from the alcoholic ammonia solution was collected, washed with water and dried, yield 9.0 g., m.p. 273°. This amidine is very sparingly soluble in boiling water, one part requiring 2000 parts for solution; it then separated with m.p. 282° (efferv.). The main bulk of the base (9.0 g.) was dissolved in 2*N*-hydrochloric acid (11.8 c.c.) and water (20 c.c.) with the aid of heat, and on cooling *p-sulphon-(p'-N'-dimethylsulphamylphenyl)amidobenzamidine hydrochloride* separated in tablets, yield 9.8 g. For analysis it was recrystallized from water (15 c.c.) and separated as a hydrate, m.p. 170° (efferv.), resolidifying on further heating and then melting at about 240°. (Found: C, 41.5; H, 4.8; H_2O , 4.6. $C_{15}H_{18}O_4N_4S_2$, HCl , H_2O requires C, 41.2; H, 4.8; H_2O , 4.1 %.)

p-Sulphon-(p'-dimethylsulphamylphenyl)amidobenzamidoxime. *p*-Cyanobenzenesulphonamidobenzenesulphondimethylamide (9.1 g.) was converted into the amidoxime in the usual way. The alcohol was removed, water added and an oil separated which solidified on keeping, yield 9.8 g. It was crystallized from boiling water (1400 c.c.) and separated in long needles, m.p. 118° (efferv.), but when dried at 100° it sintered at 127–130° and effervesced at about 160° without formation of a meniscus. (Found: C, 42.6; H, 4.7; N, 13.0; loss at 100°, 4.3. $C_{15}H_{18}O_5N_4S_2$, $1\frac{1}{2}H_2O$ requires C, 42.3; H, 5.0; N, 13.2; H_2O , 6.4 %). Attempts to prepare a homogeneous crystalline hydrochloride were not successful.

Amidines containing sulphonic or sulphinic acid groups

p-Sulphobenzamidine. Potassium *p*-cyanobenzenesulphonate (5.5 g.) was suspended in absolute alcohol (7.2 c.c.) and then subjected to the usual stages for amidine formation. The crystalline solid which separated from the ammoniacal solution was collected, washed with water to remove ammonium chloride and proved to be *p-sulphobenzamidine* (4.15 g.). On crystallization from 200 parts of boiling water it separated in small rectangular plates, m.p. above 370°. (Found: C, 42.0; H, 4.2; N, 13.8. $C_7H_5O_3N_3S$ requires C, 42.0; H, 4.0; N, 14.0 %.) It gave the amidine colour reaction described by Fuller (1944).

p-Sulphinobenzamidine hydrochloride. (a) From *p*-cyanobenzenesulphonhydroxylamide. Sodium (4.08 g.) was allowed to react with absolute alcohol (56 c.c.) and the solution was then added gradually to a hot solution of hydroxylamine hydrochloride (12.24 g.) in water (5.6 c.c.), avoiding boiling as far as possible. The solution was cooled, filtered from sodium chloride, and the latter washed with absolute alcohol (56 c.c.). The total filtrate was cooled in ice and treated with finely powdered *p*-cyanobenzenesulphonyl chloride (16.08 g.) in portions. The solution was kept for 12 hr. and a crop of water-soluble crystals was removed by filtration. The filtrate was evaporated to dryness below 40° and the residue treated with water (10 c.c.). The crystalline solid (6.88 g.), m.p. 169° (efferv.), was collected and crystallized by dissolving in warm methyl alcohol (8 c.c.) and adding warm water (16 c.c.). *p*-Cyanobenzenesulphonhydroxylamide separated in large plates (5.72 g.) which, recrystallized in the same way, had m.p. 178° (efferv.). (Found: C, 42.7; H, 3.4; S, 16.6. $C_7H_6O_3N_2S$ requires C, 42.4; H, 3.1; S, 16.2 %.) When crystallized from hot water this substance gradually decomposed with evolution of brown fumes. The pure substance gave a striking yellow colour on addition of sodium hydroxide solution.

The filtrate from the final crystallization deposited feathery needles (0.3 g.) which evolved bubbles of gas on boiling with water. It could be crystallized readily from methyl alcohol by addition of water. When, however, it was boiled with methyl alcohol (3 c.c.) it seemed to become less soluble and then separated in well-formed tablets, m.p. 138° (efferv.), yield 0.27 g. These readily effloresced in the air and became opaque, probably through loss of methyl alcohol of crystallization; they gave no yellow colour with sodium hydroxide solution. They proved, on analysis, to be *NN*-di-*p*-cyanobenzenesulphonhydroxylamide. (Found: C, 46.3; H, 2.4; S, 17.9. $C_{14}H_6O_6N_4S_2$ requires C, 46.3; H, 2.5; S, 17.7 %.) The constitution of this substance was also proved by synthesis. Thus, sodium *p*-cyanobenzenesulphinate (3.5 g.) dissolved in water (35 c.c.) was treated with sodium nitrite (0.8 g.) and 3*N*-hydrochloric acid added drop-wise with stirring until the reaction of the solution was just acid to Congo-paper. The solid which separated was collected, yield 3.2 g., m.p. 130° (efferv.). It crystallized from methyl alcohol (30 c.c.) in large tablets or columns, m.p. 138° (efferv.), which readily effloresced in the air. A mixed m.p. with the above described *NN*-di-*p*-cyanobenzenesulphonhydroxylamide showed no depression.

For the preparation of *p*-sulphinobenzamidine, *p*-cyanobenzenesulphonhydroxylamide (6.0 g.) was dissolved in absolute alcohol (25 c.c.) and converted into the imino-ether hydrochloride and then into the amidine in the usual way. The alcoholic ammonia solution deposited a white solid (7.6 g.) which contained a trace of chloride. When crystallized from *N*-hydrochloric acid (65 c.c.), the free base separated (3.75 g.) in stout prisms or tablets, m.p. 310–320°. (Found: C, 45.3; H, 4.5; S, 17.5. $C_7H_5O_2N_2S$ requires C, 45.6; H, 4.4; S, 17.4 %.) This amidine is only very weakly basic, is soluble in sodium hydroxide, but not in sodium bicarbonate solution. It gives Fuller's amidine colour reaction. When dissolved in warm 3*N*-hydrochloric acid or stronger acid, crystals separated on cooling as fine needles, m.p. above 350°,

which proved, on analysis, to be *p*-sulphinobenzamidine hydrochloride. (Found: C, 38.0; H, 4.3. $C_7H_8O_2N_2S$, HCl requires C, 38.1; H, 4.1 %.)

(b) From *p*-cyanobenzenesulphinic acid. *p*-Cyanobenzenesulphonyl chloride (20 g.) was finely powdered and stirred with sodium sulphite heptahydrate (60 g.) and water (130 c.c.) for 2 hr., the pH being maintained throughout at 7–8 by addition of sodium hydroxide solution. The solution was cooled in the ice-chest and then acidified strongly with 60 % sulphuric acid. The precipitated *p*-cyanobenzenesulphinic acid was collected, suspended in water (40 c.c.) and again collected. The product (12.62 g.) was readily soluble in water and crystallization from water gave fine colourless prisms, m.p. 126–127°. (Found: C, 50.7; H, 3.2. $C_7H_5O_2NS$ requires C, 50.3; H, 3.0 %.)

p-Cyanobenzenesulphinic acid (6.3 g.) was suspended in absolute alcohol (20 c.c.) and the process of amidine formation carried through in the customary manner. The white crystalline deposit from the alcoholic ammonia was collected and a second crop obtained by concentrating the mother liquor. Finally, the alcohol was completely removed and the residue treated with saturated sodium bicarbonate solution which precipitated a solid. This, together with the first two crops freed from ammonium chloride by washing, amounted to 6.05 g. It was dissolved in almost boiling *N*-hydrochloric acid (37 c.c.) and gave *p*-sulphinobenzamidine (3.73 g.), m.p. 310–320° with some decomposition. A mixture with the same substance prepared by the alternative method described above behaved similarly. (Found: C, 45.6; H, 4.4. Calc. for $C_7H_8O_2N_2S$: C, 45.6; H, 4.4 %.)

Aliphatic sulphonamido-amidines

ζ -Sulphonamido ϵ nanthamidine hydrochloride. 6-Chlorohexyl alcohol was prepared in 47 % yield by an adaptation of Bennett & Mosses's (1931) modification of Alberti & Smieciuszewski's method (1903, 1906). Hexamethylene glycol (30.7 g.) was mixed with cooling with concentrated hydrochloric acid (300 c.c., 32 %), the temperature then raised to 95° and the chlorohydrin extracted by a fairly rapid stream of toluene in an apparatus such as that described by Bennett and Mosses. Mechanical stirring to ensure rapid removal of the chlorohydrin as it is formed is an advantage.

The chlorohydrin (18.2 g.) was boiled for 30 hr. with a solution of sodium cyanide (10 g.) in water (20 c.c.) and alcohol (100 c.c.). The solvent was removed, water added and the 6-cyanoheptyl alcohol was extracted with ether and fractionated, yield 89.4 %, b.p. 151° at 14 mm. (Found: C, 66.1; H, 10.5; N, 10.9. $C_7H_{13}ON$ requires C, 66.1; H, 10.3; N, 11.0 %.) This was converted into 6-cyanoheptyl bromide by adding phosphorus tribromide (13.5 g.) drop-wise to the alcohol (20 g.) and heating the mixture on the water-bath for 4 hr. Water was added, an ethereal extract made and the residue obtained on removal of the ether was fractionally distilled, yield 76.9 %, b.p. 140–141° at 14 mm. (Found: N, 7.3. $C_7H_{13}NBr$ requires N, 7.4 %.)

Sodium 6-cyanoheptylsulphonate was prepared by boiling a solution of sodium sulphite heptahydrate (27.45 g.) in water (38 c.c.) with the cyanoheptyl bromide

(20.7 g.) until uniform solution was effected (5 hr.). After removal of most of the water, spirit was added and a succession of crops of crystalline material obtained (27.2 g. in all), which on crystallization from boiling spirit (350 c.c.) gave the pure sodium salt (20.3 g., 87.6 % yield) as glistening scales. (Found: C, 39.7; H, 5.4; N, 6.7; Na, 10.9. $C_7H_{12}O_3NSNa$ requires C, 39.4; H, 5.7; N, 6.6; Na, 10.8 %.)

For the preparation of 6-cyanoheoxylsulphonamide, the previously described sodium sulphonate (5.3 g.) was heated on the water-bath with phosphorus pentachloride (5.2 g.) for 1 hr. The phosphorus oxychloride was distilled off and a benzene extract of the residue was added in portions to an ammonia solution (50 c.c., d 0.88) cooled to 0° and vigorously shaken after each addition. As there was no separation of solid, the solvents were completely removed and the desired product obtained by extraction of the residual salts with acetone. The final acetone solution on removal of the solvent left a syrup which gradually crystallized in a freezing mixture, yield 5.2 g. It was distilled at 250° (external bath temperature), at 1 mm., dissolved in acetone to free it from a persistent amorphous impurity and, on concentration to a very small volume of solvent, readily crystallized, m.p. 49°. It could now be crystallized from 25 vol. of boiling water and separated in clusters of prisms, m.p. 49–50°. (Found: C, 44.6; H, 7.0; N, 14.7. $C_7H_{14}O_2N_2S$ requires C, 44.2; H, 7.4; N, 14.7 %.)

For amidine formation the crude cyanoheoxylsulphonamide (from 4.3 g. of sodium 6-cyanoheoxylsulphonate) was dissolved in absolute ethyl alcohol (5 c.c.) and the process carried through in the usual manner. The alcoholic ammonia, showing no deposit, was evaporated to dryness and the residue treated with alcohol to remove ammonium chloride. On concentration, crystallization was effected by addition of dry ethyl acetate to the alcoholic solution. ζ -Sulphonamidobenanthamidine hydrochloride (3.38 g.) so obtained separated from absolute ethyl alcohol in crusts of needles, m.p. 127–128°. (Found: C, 34.8; H, 7.3. $C_7H_{17}O_2N_3S$, HCl requires C, 34.5; H, 7.4 %.)

Anhydro- γ -sulphobutyramidine (XVIII). γ -Chlorobutyronitrile (30 g.) (Allen, 1928) was refluxed with an aqueous solution (120 c.c.) of sodium sulphite heptahydrate (75 g.) for $\frac{3}{4}$ hr. and the clear solution was then evaporated to dryness. The dry residue was thoroughly extracted in a continuous extractor with hot isopropyl alcohol and sodium γ -cyanopropene- α -sulphonate separated in glittering plates, m.p. 234–235°. (Found: N, 8.2. $C_4H_6O_3NSNa$ requires N, 8.2 %.)

The above sodium salt (17.1 g.) was intimately mixed with powdered phosphorus pentachloride (20.8 g.), when an immediate exothermic reaction took place accompanied by liquefaction of the mixture. After standing for 1.5 hr. the mixture was treated with ice-water and the oil which separated extracted with ether. The ether solution, dried over sodium sulphate, was mixed with 10 % alcoholic ammonia solution and set aside for 2 hr. The precipitated ammonium chloride was filtered off and the filtrate evaporated to dryness under reduced pressure below 40°. The residue was extracted with ethyl acetate and the filtered extract evaporated to dryness left a colourless syrup (5.6 g.). Miller *et al.* (1940) obtained γ -cyanopropene- α -sulphonamide as a solid, m.p. 65–66°.

The syrup (5.3 g.) was dissolved in absolute alcohol (9 c.c.) and imino-ether hydrochloride and amidine formation carried out in the usual way. The white crystalline solid was collected (2.3 g., m.p. 210–220°) and washed with a little alcohol and ether. It was suspended in water (4.5 c.c.) and filtered. It was resuspended in water, giving a suspension with pH 7.6; as one drop of 2*N*-hydrochloric acid brought the pH below 4, the substance was obviously not free amidine base. Crystallization from water afforded large colourless prisms of *anhydro-γ-sulphobutyramidine*, m.p. 236–237°. (Found: Loss at 100° in a vacuum, 14.6. $C_4H_8O_2N_2S$, $1\frac{1}{2}H_2O$ requires H_2O , 15.4 %. Found on dried material: C, 32.5; H, 5.5; N, 19.3; S, 21.7. $C_4H_8O_2N_2S$ requires C, 32.4; H, 5.4; N, 18.9; S, 21.6 %.)

Amidoximes containing the groups $-SO_2NH_2$, $-SO_2NHR$,
 $-SO_2NRR'$ and $-SO_2C\leq$

p-*Sulphonamidobenzamidoxime hydrochloride* (II). Anhydrous sodium carbonate (26.5 g.) was dissolved in water (100 c.c.) with slight warming and hydroxylamine hydrochloride (25 g.) added. *p*-*Sulphonamidobenzonitrile* (91 g.) was then added followed by ethyl alcohol (200 c.c.). The mixture was boiled on the water-bath for 1.5 hr., separation of amidoxime often taking place within 10 min. of the commencement of boiling. The condenser was reversed and about 190 c.c. of aqueous alcohol distilled off within 30 min. The residue was diluted with water (100 c.c.), cooled and filtered, yield 85–93 g. *p*-*Sulphonamidobenzamidoxime* was obtained pure by crystallization from 10 vol. of boiling water, yield 75–88 g., m.p. 210° (efferv.). (Found: C, 38.9; H, 4.1; N, 19.2. $C_7H_9O_3N_3S$ requires C, 39.0; H, 4.2; N, 19.5 %.) This base was dissolved in the calculated amount of 2*N*-hydrochloric acid and the solution concentrated slightly under reduced pressure. *p*-*Sulphonamidobenzamidoxime hydrochloride hemihydrate* separated in needles, m.p. 152–153° (efferv.). Occasionally considerable shrinkage may occur about 150°, followed by m.p. 201° with effervescence. When a specimen shows the first m.p. at 153°, it resolidifies in needles and then melts at 201° (efferv.). From more dilute solution it separates in well-formed rods or prisms. (Found: C, 32.2; H, 4.3; N, 16.2; Cl, 13.6; H_2O , 3.6. $C_7H_9O_3N_3S$, HCl, $\frac{1}{2}H_2O$ requires C, 32.2; H, 4.3; N, 16.1; Cl, 13.6; H_2O , 3.5 %.) It gives Fuller's amidine colour reaction after reduction in acid solution with zinc dust.

In preparing large quantities of *p*-sulphonamidobenzamidoxime, small quantities of *p*-cyanobenzenesulphonamide, *p*-carbamylbenzenesulphonamide and a new substance 3:5-di-*p*-sulphonamidophenyl-1:2:4-oxdiazole were isolated from the final mother liquors. This oxdiazole, m.p. 313°, is soluble in 430 parts of boiling glacial acetic acid and separates in long needles. It gave a crystalline sodium salt on treatment with 2*N*-sodium hydroxide but was unaffected by sodium carbonate or ammonia solution. (Found: C, 44.4; H, 3.2; N, 14.7. $C_{14}H_{13}O_6N_4S_2$ requires C, 44.2; H, 3.2; N, 14.7 %.) The formation of this substance is paralleled by the formation of small quantities of 3:5-diphenyl-1:2:4-oxdiazole during the preparation of benzamidoxime from benzonitrile.

4-Sulphonamido-3-methylbenzamidoxime. 3-Cyanotoluene-6-sulphonamide (4.9 g.) was treated with hydroxylamine hydrochloride (1.75 g.) and anhydrous sodium carbonate (1.33 g.) in water (5 c.c.) and sufficient alcohol (20 c.c.) was added to afford a homogeneous solution at the boiling-point. After refluxing for 3 hr. the solvent was removed and water (10 c.c.) was added. The solid (5.0 g.) so obtained separated from water in clusters of colourless hard prisms, m.p. 183–184° (efferv.). (Found: C, 41.9; H, 4.9; N, 18.3. $C_8H_{11}O_3N_3S$ requires C, 41.9; H, 4.8; N, 18.3 %.) The hydrochloride was obtained by neutralizing the free amidoxime with the calculated volume of *N*-hydrochloric acid, followed by evaporation to dryness. Crystallization from a small volume of water afforded fine colourless needles, m.p. 223° (efferv.). (Found: C, 36.6; H, 4.7; N, 15.6. $C_8H_{11}O_3N_3S$, HCl requires C, 36.2; H, 4.5; N, 15.8 %.)

***p*-Sulphondiethylamidobenzamidoxime hydrochloride.** *p*-Cyanobenzenesulphondiethylamide (4.76 g.) and equivalent amounts of hydroxylamine hydrochloride and anhydrous sodium carbonate in aqueous alcohol (10 c.c. : 15 c.c.) were boiled for 16 hr. The alcohol was removed and the oily residue converted into a solid hydrochloride. This was then reconverted to base by addition of saturated sodium bicarbonate solution, yield 2.96 g. When crystallized from alcohol *p*-sulphondiethylamidobenzamidoxime separated in clusters of well-formed prisms, m.p. 123–124°. (Found: C, 48.8; H, 6.5. $C_{11}H_{17}O_3N_3S$ requires C, 48.7; H, 6.3 %.) The base was treated with *N*-hydrochloric acid (3.6 c.c.) and water (0.4 c.c.). On warming, solution was effected and *p*-sulphondiethylamidobenzamidoxime hydrochloride (2.1 g.) separated in stout tablets or plates, m.p. 210° (efferv.). (Found: C, 43.0; H, 6.0; N, 13.9. $C_{11}H_{17}O_3N_3S$, HCl requires C, 42.9; H, 5.9; N, 13.7 %.) From the original mother liquors unchanged nitrile (0.55 g.) was recovered by extraction with ether.

***p*-Sulphondi- β -hydroxyethylamidobenzamidoxime hydrochloride.** *p*-Cyanobenzenesulphondi- β -hydroxyethylamide (6.6 g.) was converted into the amidoxime in the usual way. The solvent was completely removed and the absolute ethyl alcoholic extract of the dried residue on concentration gave *p*-sulphondihydroxyethylamidobenzamidoxime (2.66 g.). On recrystallization, twice, from 30 parts of boiling alcohol, it separated in needles, m.p. 183–184°. (Found: C, 43.5; H, 5.5; N, 14.2. $C_{11}H_{17}O_5N_3S$ requires C, 43.5; H, 5.6; N, 13.9 %.) The base (2.0 g.) was dissolved in 2*N*-hydrochloric acid (3.3 c.c.) and, on slight concentration and cooling to 0°, *p*-sulphon- β -dihydroxyethylamidobenzamidoxime hydrochloride (1.82 g.) separated in needles, m.p. 172–173° (efferv.). (Found: C, 38.6; H, 5.2. $C_{11}H_{17}O_5N_3S$, HCl requires C, 38.9; H, 5.3 %.)

***p*-Sulphonpiperididobenzamidoxime hydrochloride.** *p*-Cyanobenzenesulphonpiperidide (5.0 g.) was converted into the amidoxime as usual. During the boiling the base crystallized out and, on removal of the alcohol, was collected, yield 5.29 g. On crystallization from boiling alcohol (150 c.c.) *p*-sulphonpiperididobenzamidoxime separated in spiked needles, m.p. 196–197°. (Found: C, 51.4; H, 6.2; N, 14.9. $C_{12}H_{17}O_3N_3S$ requires C, 50.9; H, 6.1; N, 14.8 %.) The hydrochloride (0.83 g.) was obtained by solution of the base (2.25 g.) in the calculated amount of 2*N*-hydrochloric acid (4 c.c.) and concentration of the solution to about one-third of its volume. It

then separated in thin diamond-shaped plates, m.p. about 260° with decomposition. (Found: C, 45.3; H, 5.7; N, 13.0. $C_{12}H_{17}O_3N_3S$, HCl requires C, 45.1; H, 5.7; N, 13.1 %.)

p-Sulphonmorpholidobenzamidoxime hydrochloride. *p*-Cyanobenzenesulphonmorpholide (8.4 g.) was converted into the amidoxime. On removal of the alcohol it separated as an oil which soon solidified, yield 8.8 g. On crystallization from alcohol (20 c.c.), *p-sulphonmorpholidobenzamidoxime* separated in blades or plates, m.p. $158-159^{\circ}$ (efferv.). (Found: C, 46.7; H, 5.3; N, 14.6. $C_{11}H_{15}O_4N_3S$ requires C, 46.3; H, 5.3; N, 14.7 %.) The *hydrochloride* crystallized readily in the form of needles on dissolving the base (2.85 g.) in 2*N*-hydrochloric acid (5.5 c.c.). A sample of these needles, on heating in a capillary tube, shrank at about 240° , then melted and decomposed at about 270° . (Found: C, 39.6; H, 5.4; N, 12.4; loss at 110° , 4.5. $C_{11}H_{15}O_4N_3S$, HCl, $\frac{1}{2}H_2O$ requires C, 39.9; H, 5.2; N, 12.7; H_2O , 2.7 %.)

p-Hydroxyguanylbzenesulphonamidothiazole hydrochloride. *p*-Cyanobenzenesulphonamidothiazole (8.83 g.) was converted into the amidoxime in aqueous alcohol solution. On removal of the alcohol, *p-hydroxyguanylbzenesulphonamidothiazole* was collected, yield 8.76 g. It was crystallized from boiling water (2250 c.c.) and separated in leaflets, m.p. 223° (efferv.). (Found: C, 39.9; H, 3.3; N, 18.4. $C_{10}H_{10}O_3N_4S_2$ requires C, 40.2; H, 3.4; N, 18.8 %.) The *hydrochloride* was prepared by solution of the base in the calculated amount of 2*N*-hydrochloric acid and adding a slight excess of 32 % hydrochloric acid. It separated in microscopic tablets, m.p. 218° (efferv.). (Found: C, 35.6; H, 3.6. $C_{10}H_{10}O_3N_4S_2$, HCl requires C, 35.9; H, 3.3 %.)

p-Sulphonhydroxylamidobenzamidoxime (IV). A warm solution of sodium (2.3 g.) in absolute ethyl alcohol (32 c.c.) was added to an almost boiling solution of hydroxylamine hydrochloride (7.0 g.) in water (2 c.c.) at such a rate as to avoid boiling and yet fast enough to avoid separation of hydroxylamine hydrochloride. When the solution was cold, sodium chloride was removed and absolute alcohol (32 c.c.) added followed by *p*-cyanobenzenesulphonyl chloride (5.0 g.) in portions. Solution occurred rapidly with evolution of gas and on keeping overnight a water-soluble crop of crystals (1.88 g.) was removed. The alcoholic mother liquor was evaporated to dryness below 50° and the residue treated with water (10 c.c.), when *p-sulphonhydroxylamidobenzamidoxime monohydrate* (3.22 g.) separated. From boiling water (20 c.c.) it crystallized in needles, m.p. $152-153^{\circ}$ (efferv.). (Found: C, 34.0; H, 4.4; N, 16.8. $C_7H_5O_4N_3S$, H_2O requires C, 33.7; H, 4.4; N, 16.9 %.) An aqueous solution gave an evanescent purple colour with ferric chloride solution. The mother liquors on keeping deposited a white solid (0.17 g.) which crystallized after solution in boiling water in fine needles, m.p. 295° (decomp.). This proved to be *p-sulphonhydroxylamidobenzamide*. (Found: C, 38.9; H, 3.8; N, 13.0. $C_7H_5O_4N_3S$ requires C, 38.9; H, 3.7; N, 13.0 %.)

p-Sulphon-O-methylhydroxylamidobenzamidoxime hydrochloride. *p*-Cyanobenzenesulphon-*O*-methylhydroxylamide (4.24 g.) was converted into the amidoxime in the usual way. The product (4.62 g.) crystallized out on removing the alcohol; it was

dissolved in *N*-hydrochloric acid (20 c.c.) and the solution on concentration deposited needles of *p*-sulphon-*O*-methylhydroxylamidobenzamidoxime hydrochloride (3.16 g.), m.p. 198° (efferv.). (Found: C, 33.8; H, 4.5; N, 14.6. $C_8H_{11}O_4N_3S$, HCl requires C, 34.1; H, 4.3; N, 14.9 %.)

p-Sulphon-*O*-ethylhydroxylamidobenzamidoxime hydrochloride. *p*-Cyanobenzene-sulphon-*O*-ethylhydroxylamide (4.52 g.) was converted into the amidoxime. The crude base (4.3 g.) was ground with *N*-hydrochloric acid (16.6 c.c.) and, on concentration of the solution after removal of a sparingly soluble solid (0.52 g.), *p*-sulphon-*O*-ethylhydroxylamidobenzamidoxime hydrochloride separated in small needles (3.36 g.), m.p. 151–152°. (Found: C, 36.0; H, 4.9; N, 13.5; H_2O , 4.8. $C_9H_{13}O_4N_3S$, HCl, $\frac{1}{2}H_2O$ requires C, 35.5; H, 5.0; N, 13.8; H_2O , 3.0 %.) The sparingly soluble solid (above) was crystallized from water (90 c.c.) and separated in fern-like crystals (0.15 g.), m.p. 215–216°, which proved to be *p*-sulphon-*O*-ethylhydroxylamidobenzamide. (Found: C, 44.3; H, 4.8; N, 11.4. $C_9H_{12}O_4N_2S$ requires C, 44.2; H, 4.9; N, 11.5 %.)

p-Sulphon-*O*-propylhydroxylamidobenzamidoxime hydrochloride. *p*-Cyanobenzene-sulphon-*O*-propylhydroxylamide (4.8 g.) was converted into the crude amidoxime base (5.82 g.). This was ground with *N*-hydrochloric acid (21.3 c.c.) and filtered from unchanged nitrile (1.03 g.). The filtrate on concentration deposited *p*-sulphon-*O*-propylhydroxylamidobenzamidoxime hydrochloride as needles (3.41 g.), m.p. 145–150° (efferv.). (Found: C, 38.1; H, 5.4; N, 13.1; H_2O , 3.7. $C_{10}H_{15}O_4N_3S$, HCl, $\frac{1}{2}H_2O$ requires C, 37.7; H, 5.4; N, 13.2; H_2O , 2.8 %.)

p-Sulphon-*O*-butylhydroxylamidobenzamidoxime hydrochloride. *p*-Cyanobenzene-sulphon-*O*-butylhydroxylamide (5.08 g.) was converted into the crude amidoxime base (6.2 g.). This was treated with *N*-hydrochloric acid (21.6 c.c.), filtered from unchanged nitrile (0.63 g.) and the solution concentrated. On standing, needles of *p*-sulphon-*O*-butylhydroxylamidobenzamidoxime hydrochloride (4.89 g.) separated. For analysis it was recrystallized from its own weight of water and then melted about 130°, but only formed a meniscus at about 140°. (Found: C, 40.0; H, 5.7; N, 12.4. $C_{11}H_{17}O_4N_3S$, HCl, $\frac{1}{2}H_2O$ requires C, 39.7; H, 5.8; N, 12.6 %.)

p-Methylsulphonylbenzamidoxime. *p*-Methylsulphonylbenzonitrile (4.5 g.), hydroxylamine hydrochloride (1.75 g.) and anhydrous sodium carbonate were refluxed in 65 % aqueous alcohol for 1.75 hr. and a considerable separation of colourless prisms took place during this time. The alcohol was distilled off and a small volume of water was added. The product (5.2 g.) crystallized from water in colourless, long, thin, hard prisms, m.p. 188° (decomp.). (Found: C, 44.9; H, 4.8; N, 13.0. $C_8H_{10}O_3N_2S$ requires C, 44.8; H, 4.7; N, 13.1 %.)

Sulphonamidobenzamidoximes substituted in the amidoxime group

p-Sulphonamidobenzamidoxime-*O*-methyl ether hydrochloride and *NN'*-dimethoxybenzamidine-*p*-sulphonamide. *p*-Sulphonamidobenziminoethyl ether hydrochloride (10 g.) prepared in the usual way was added to absolute alcohol (60 c.c.) in a pressure bottle and then *O*-methylhydroxylamine base (18 c.c.) added all at once. A clear

solution was immediately obtained with evolution of heat. After keeping at 37° for a fortnight, ammonium chloride (1.21 g.) was removed; on evaporation to a syrup and addition of water and complete removal of alcohol, and on further addition of water, a solid (9.3 g.) was precipitated. This was divided by treatment with cold *N*-hydrochloric acid into an acid-insoluble portion (2.3 g.) and an acid-soluble fraction. The former on crystallization from water (35 c.c.) separated in plates, m.p. 145–146°, and this m.p. was unchanged on recrystallization from water or chloroform. Analysis of the product from either solvent showed it to be *NN'*-dimethoxybenzamidine-*p*-sulphonamide. (Found: C, 41.6; 41.8; H, 5.0, 5.0; N, 16.2, 15.9. $C_9H_{13}O_4N_3S$ requires C, 41.7; H, 5.0; N, 16.2 %.) When an attempt was made to estimate the methoxyl groups in this substance by Zeisel's method (macro or micro), the methyl iodide was only evolved very slowly and low results were obtained. (Found: OMe (macro) 17.5; (micro) 17.1, 17.6. $C_9H_{13}O_4N_3S$ requires 2-OMe, 23.9 %.) In this connexion it can be quoted that Kaufler found that methyl iodide was given off slowly from phenylazoacetaldoxime-*O*-methyl ether and that low results for *O*-methyl were obtained (Bamberger & Frei 1902). This dimethoxybenzamidine-*p*-sulphonamide is weakly basic, dissolving in 3*N*-hydrochloric acid and forming an oily nitroso-derivative on addition of nitrite. A certain amount is soluble in *N*-hydrochloric acid, but is largely precipitated on dilution. The main *N*-hydrochloric acid extract above was therefore diluted with water to remove slightly basic material and carefully concentrated *in vacuo* over sulphuric acid. By fractional crystallization *p*-sulphonamidobenzamidoxime-*O*-methyl ether hydrochloride (2.82 g.) was obtained in plates, m.p. 214–215°. (Found: C, 36.4; H, 4.6; N, 15.8. $C_8H_{11}O_3N_3S$, HCl requires C, 36.1; H, 4.6; N, 15.8 %.) From the fraction precipitated by water *p*-sulphonamidobenzoic acid, m.p. 295°, was isolated by fractional crystallization. (Found: C, 41.7; H, 3.8; N, 7.3. Calc. for $C_7H_7O_4NS$: C, 41.8; H, 3.5; N, 7.0 %.)

p-Sulphonamidobenzamidoxime-*O*-ethyl ether hydrochloride and *NN'*-diethoxybenzamidine-*p*-sulphonamide. *p*-Sulphonamidobenziminoethyl ether hydrochloride (10 g.) was suspended in absolute alcohol (50 c.c.) and *O*-ethylhydroxylamine (20 c.c.) added. The pressure bottle was sealed and kept for 14 days at 37°. After removal of ammonium chloride the liquor was evaporated to dryness. The residue was treated with a little water and the solid (11.35 g.) collected. This was ground up with *N*-hydrochloric acid (35 c.c.) leaving a solid (5.88 g.) undissolved. The latter was crystallized twice from benzene from which it separated in plates (5.4 g.) which readily effloresced and disintegrated. One more crystallization from water (170 c.c.) gave needles (3.42 g.) of *NN'*-diethoxybenzamidine-*p*-sulphonamide, m.p. 130–131°. (Found: C, 46.5; H, 5.9. $C_{11}H_{17}O_4N_3S$ requires C, 46.6; H, 5.9 %.) The aqueous mother liquor of this sulphonamide on concentration deposited a crude solid (1.69 g.) which on boiling with chloroform left a substance (0.55 g.) undissolved and which on crystallization from water (5 c.c.) gave well-formed prisms (0.5 g.) of *p*-sulphonamidobenzamidoxime-*O*-ethyl ether, m.p. 152–153°. A further comparable quantity was obtained by reworking the mother liquors. (Found: C, 44.2; H, 5.4; N, 17.1. $C_9H_{13}O_3N_3S$ requires C, 44.4; H, 5.4; N, 17.3 %.)

The N-hydrochloric acid-soluble fraction (above) on concentration and fractional crystallization gave *p*-sulphonamidobenzamidoxime-O-ethyl ether hydrochloride, m.p. 221–222° (efferv.), yield 2.1 g. (Found: C, 38.7; H, 5.1; N, 14.7. $C_9H_{14}O_3N_3S$, HCl requires C, 38.6; H, 5.0; N, 15.0 %.)

p-Sulphonamidobenzamidoxime-O-propyl ether hydrochloride and NN'-dipropoxybenzamidine-*p*-sulphonamide. *p*-Sulphonamidobenziminoethyl ether hydrochloride (6.6 g.) suspended in absolute alcohol (40 c.c.) was treated with *O*-propylhydroxylamine (10.5 g.) and kept at 37° in a pressure bottle for 70 days. The solvent was completely removed and the residue treated with a little water, which left a solid undissolved (6.83 g.). This was extracted with N-hydrochloric acid (26.5 c.c.) and the base (5.13 g.) liberated by sodium bicarbonate. It separated from 75 parts of boiling benzene in filmy leaflets, m.p. 127–128°, which readily effloresced and became dull in appearance. Analysis showed it to be *p*-sulphonamidobenzamidoxime-O-propyl ether containing one-half a molecule of benzene of crystallization. (Found on freshly crystallized specimen: Loss at 95°, 13.1. $C_{10}H_{15}O_3N_3S$, $\frac{1}{2}C_6H_6$ requires loss 13.2 %. Found on dried material: C, 46.5; H, 6.3; N, 16.3. $C_{10}H_{15}O_3N_3S$ requires C, 46.7; H, 5.9; N, 16.3 %.) Of this base, a portion (2.88 g.) was treated with N-hydrochloric acid (11.2 c.c.) and on concentration the solution deposited small prisms of *p*-sulphonamidobenzamidoxime-O-propyl ether hydrochloride (2.2 g.), m.p. 178–180°. (Found: C, 40.5; H, 5.5; N, 14.5. $C_{10}H_{15}O_3N_3S$, HCl requires C, 40.9; H, 5.5; N, 14.3 %.)

The remainder of the solid, from the initial N-hydrochloric acid extraction above, was almost completely soluble in hot acid (78 c.c.) of this strength and on filtration and basification of the solution separated as a solid (1.05 g.), m.p. about 120–130° and depressed strongly by admixture with the above *p*-sulphonamidobenzamidoxime-O-propyl ether. On crystallization from boiling water in which it is sparingly soluble or from boiling benzene, NN'-dipropoxybenzamidine-*p*-sulphonamide separated in needles, m.p. 133–134°. (Found: C, 49.4; H, 6.8. $C_{13}H_{21}O_4N_2S$ requires C, 49.5; H, 6.7 %.)

p-Sulphonamidobenzamidoxime-O-butyl ether hydrochloride. *p*-Sulphonamidobenziminoethyl ether hydrochloride (5.3 g.), suspended in absolute alcohol (30 c.c.), was treated with *O*-butylhydroxylamine (16.9 g.) and kept at 37° for 14 days. The ammonium chloride was removed and the filtrate evaporated to dryness. There was no deposition of solid on adding water, so the aqueous solution was extracted with ether and the latter extracted with several portions of N-hydrochloric acid. These were combined (30 c.c.) and the solution concentrated to a small volume. *p*-Sulphonamidobenzamidoxime-O-butyl ether hydrochloride separated in plates (4.52 g.) which on crystallization from water (5.5 c.c.) had m.p. 136–137° (efferv.). (Found: C, 40.2; H, 6.1; N, 12.5. $C_{11}H_{17}O_3N_3S$, HCl, H_2O requires C, 40.5; H, 6.2; N, 12.9 %.)

p-Sulphonamidobenzureideoxime. *p*-Sulphonamidobenzamidoxime hydrochloride (2.51 g.) in water (20 c.c.) was treated with potassium cyanate (0.81 g.) also dissolved in water (5 c.c.). There was immediate separation of the ureide (2.51 g.) as clusters

of fine needles, m.p. 202° (efferv.). (Found: C, 37.2; H, 4.0. $C_8H_{10}O_4N_2S$ requires C, 37.2; H, 3.9 %.) On crystallization from water the m.p. is lowered. That the substance is a ureide and not a salt of cyanic acid follows from its recovery unchanged after treatment with sodium bicarbonate solution.

Amidoximes containing sulphonic or sulphinic acid groups

p-Sulphinobenzamidozime. *p*-Cyanobenzenesulphonyl chloride (4 g.) in warm ethyl alcohol (30 c.c.) was added in a thin stream to hydrazine hydrate (4 c.c.) in ethyl alcohol (10 c.c.), the reaction vessel being kept immersed in water at room temperature during the addition. The oil which separated soon solidified; it was collected, ground with water and dried, yield 72 %. *p*-Cyanobenzenesulphonhydrazide is soluble in 20 parts of boiling ethyl alcohol and separates in plates, m.p. 148° (efferv.). (Found: C, 43.0; H, 3.6; N, 21.1. $C_7H_7O_2N_2S$ requires C, 42.6; H, 3.6; N, 21.3 %.) It readily reduces Fehling's solution on warming.

The above hydrazide (4.93 g.) in ethyl alcohol (20 c.c.) was boiled for 3 hr. with hydroxylamine hydrochloride (1.9 g.) and anhydrous sodium carbonate (1.46 g.) with addition of water (6 c.c.). The solvent was completely removed, and sodium chloride was then removed by addition of absolute ethyl alcohol. *N*-Hydrochloric acid (25 c.c.) was then added, and alcohol with some water removed by distillation below 50° to a small volume. On keeping, small needles (1.29 g.) separated which could be recrystallized from about 100 parts of boiling water and then separated in rhombs, m.p. 236° (efferv.). Analysis showed that this solid was *p*-sulphinobenzamidozime. (Found: C, 42.2; H, 4.0; N, 14.2. $C_7H_8O_3N_2S$ requires C, 42.0; H, 4.0; N, 14.0 %.) It was only weakly basic, dissolving in 3*N*-hydrochloric acid but not in *N*-acid. In aqueous solution it gave a typical amidoxime colour reaction with ferric chloride.

In the first attempt to prepare *p*-cyanobenzenesulphonhydrazide, hydrazine sulphate (3.25 g.) was dissolved in boiling water (15 c.c.), 50 % sodium hydroxide solution (10 c.c.) added, the mixture cooled to 20° and *p*-cyanobenzenesulphonyl chloride (5.0 g.) suspended in absolute alcohol (10 c.c.) added and the mixture shaken. The clear solution kept for 12 hr. was freed from alcohol and the reaction adjusted to faint acidity. The insoluble crystalline powder (0.16 g.) which separated proved to be *NN'*-di-*p*-cyanobenzenesulphonylhydrazide. On crystallization from 44 vol. of boiling glacial acetic acid it separated in minute leaflets, m.p. 252° (efferv.). (Found: C, 46.0; H, 2.8; $C_{14}H_{10}O_4N_4S_2$ requires C, 46.4; H, 2.8 %.) This hydrazide is soluble in ammonia solution with a bright yellow colour; with 2*N*-sodium hydroxide it gives a yellow colour and a sparingly soluble sodium salt. It is soluble in warm sodium bicarbonate solution without colour development.

Miscellaneous compounds

*N*⁴-Dimethylsulphanilamide (XIX). For the preparation of *p*-dimethylaminobenzenesulphonic acid, dimethylaniline (60.5 g.) and Nordhausen sulphuric acid (50 g.) were heated to 180° for 5 hr. The required acid was first isolated as its barium

salt, the yield of acid being 28.7 g., m.p. 270°. This acid (10.0 g.), dried to constant weight at 100°, was finely powdered, mixed with phosphorus pentachloride (10.6 g.) and heated for 2 hr. on the boiling water-bath. The phosphorus oxychloride was pumped off and the residue, dissolved in benzene (30 c.c.), was added portion-wise with vigorous shaking to concentrated ammonia solution (100 c.c., d 0.88), the temperature being kept near 0°. The solid (8.9 g.) which separated overnight and that obtained on concentration were crystallized from boiling water (1200 c.c.) and separated in plates, m.p. 209–211°, which proved on analysis to be *p*-dimethylamino-benzenesulphonamide. (Found: C, 48.0; H, 6.1; N, 13.7. $C_8H_{12}O_2N_2S$ requires C, 48.0; H, 6.0; N, 14.0 %.)

*N*⁴-Dimethylsulphanilamide methiodide. The above described sulphonamide (3.07 g.) was heated in a sealed tube for 9 hr. with methyl alcohol (10 c.c.) and methyl iodide (5 c.c.) at 72°. The crystalline solid (4.0 g.) was collected, extracted with lukewarm water (30 c.c.) and the filtrate concentrated, yield 3.73 g. On recrystallization from water *N*⁴-dimethylsulphanilamide methiodide separated in pale yellow prisms or pointed tablets, m.p. 207°. (Found: C, 31.8; H, 4.3. $C_9H_{15}O_2N_2SI$ requires C, 31.6; H, 4.4 %.) The corresponding methochloride crystallized in tablets, m.p. 217° (efferv.). (Found: C, 42.9; H, 6.2. $C_9H_{15}O_2N_2SCl$ requires C, 43.1; H, 6.0 %.)

p-Sulphonamidobenzyltrimethylammonium chloride. *p*- ω -Chlorotoluenesulphonyl chloride (16.6 g., b.p. 170–190° at 17 mm.) was converted to the amide in acetone (30 c.c.) with 2*N*-ammonia solution (100 c.c.). After 1 hr. water was added and the crystalline solid collected and drained thoroughly. The crude moist product was set aside with 3.8 *N*-alcoholic trimethylamine (30 c.c.) at 37° for 3 days, and the clear light brown solution was then evaporated to dryness. The residue was dissolved in methyl alcohol (40 c.c.) and carefully treated with 3 vol. of ethyl acetate (120 c.c.), when a granular crystalline solid (11.7 g.) was precipitated, m.p. 246–248°. Recrystallization from a small volume of methyl alcohol afforded *p*-sulphonamidobenzyltrimethylammonium chloride as clusters of fine colourless prisms, m.p. 260° (efferv.). (Found: C, 45.5; H, 6.5; N, 10.6. $C_{10}H_{17}O_2N_3S$ requires C, 45.4; H, 6.4; N, 10.6 %.)

p-Sulphonamidophenylguanidine hydrochloride (XVI). Sulphanilamide hydrochloride (6.2 g.) and cyanamide (2.5 g.) were boiled in alcoholic solution for 21 hr. The alcohol was removed and saturated sodium picrate solution added until there was no further precipitation. The crystalline picrate (8.2 g.) was collected, crystallized twice from 12 parts of boiling water and separated in hexagonal plates, m.p. 206–207°. (Found: C, 35.5; H, 3.0; N, 21.8. $C_7H_{10}O_2N_4S$, $C_6H_5O_7N_3$ requires C, 35.2; H, 3.0; N, 22.1 %.) The picrate (6.3 g.) was converted into the hydrochloride of *p*-sulphonamidophenylguanidine (3.65 g.). On drying at 90° it melted at 118–119°, but on prolonged drying at 115° it softened at 151° and then melted at 188°. (Found: C, 31.6; H, 5.0; N, 20.4. $C_7H_{10}O_2N_4S$, HCl, H_2O requires C, 31.3; H, 4.9; N, 20.8 %.) An attempt to isolate the hydrochloride without proceeding through the picrate was not successful.

NN'-Di-*p*-cyanobenzenesulphonylguanidine. The preparation of *p*-cyanobenzenesulphonylguanidine from the acid chloride was not successful, since orthodox

methods gave no water-insoluble product through hydrolysis of the sulphonyl chloride with formation of the guanidine salt (compare Karrer & Epprecht 1941). By use of the precise conditions described for the preparation of *p*-acetamidobenzenesulphonylguanidine (Bratton *et al.* 1940) a disubstituted guanidine was obtained.

Finely powdered guanidine nitrate (12.2 g.), water (19 c.c.), powdered sodium hydroxide (10 g.) and acetone (40 c.c.) were treated with *p*-cyanobenzenesulphonyl chloride (20.15 g.) in acetone (80 c.c.). After an hour 7 vol. of water were added to the sludge and the white amorphous solid was collected, washed with a little water and dried. The substance (14.8 g.), m.p. 266–274° (efferv.) after sintering at 250°, appeared to be a salt; it was moderately soluble in warm water and addition of hydrochloric acid gave a precipitate of the free acid. The latter was sparingly soluble and crystallization from glacial acetic acid, or 60% aqueous alcohol, gave fine silky colourless needles of NN'-di-*p*-cyanobenzenesulphonylguanidine, m.p. 255–258°. (Found: C, 46.1; 46.3, H, 3.0, 3.0; N, 17.6; S, 16.6. $C_{15}H_{11}O_4N_5S_2$ requires C, 46.3; H, 2.8; N, 18.0; S, 16.5%.) The substance was not soluble in camphor and di- amidine formation was not attempted.

p-Sulphonamidobenzhydroxamic acid. A solution of free hydroxylamine in methyl alcohol was prepared from hydroxylamine hydrochloride (5.6 g.) and excess potassium hydroxide (6.73 g.) (Hauser & Renfrow 1939). To the methyl alcoholic solution (total volume 70 c.c.) was added ethyl *p*-sulphonamidobenzoate (9.1 g.) in dioxan (25 c.c.) and the mixture kept at room temperature for 4 days. The solid was collected, washed with absolute methyl alcohol and dried. The crude potassium salt (4.65 g.) was treated with the calculated volume of 1.25 N-acetic acid on the water-bath and on cooling a thick paste of crystals was obtained. The solid was collected, dried and extracted with cold ethyl acetate. The crude product (3.5 g., m.p. 145°) separated from 75% aqueous alcohol as a mass of felted needles (2.3 g.), m.p. 178°. (Found: C, 38.7; H, 3.5; N, 12.9. $C_7H_8O_4N_2S$ requires C, 39.0; H, 3.7; N, 12.9%.) The substance gave a blood-red colour with ferric chloride solution.

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Photochemical laws and visual phenomena

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By measuring the course of recovery of visual sensitivity in the dark, after exposure to light, it has been demonstrated that all the phenomena of recovery can be explained—qualitatively—by the known fundamental principles of photochemistry. In particular, the reciprocity law, which is widely valid in photographic processes, only holds under certain limited circumstances for visual phenomena. In the case of foveal vision the reciprocity law does not hold, owing to the rapid recovery process, except for very brief periods of exposure. In the case of parafoveal vision the recovery process for the scotopic mechanism is sufficiently slow to allow validity of the reciprocity law over a wide range of exposure periods, although the law still breaks down for the parafoveal photopic mechanism. This circumscribed validity of the reciprocity law is of practical service in that the effect of many experimental conditions can be predicted from a very limited set of suitably chosen experimental data. It may be noted that it has not been found necessary to introduce any postulate involving recovery of the nervous transmission system as part of the recovery process.

1. INTRODUCTION

It is generally accepted that a visual sensation is produced by a photochemical reaction in the retina, the secondary effects of this being transmitted by nervous action to the brain. It follows that the recovery of the state of dark adaptation after exposure of the eye to light may be due to a reverse reaction after the photochemical decomposition, to replacement of the supply of photosensitive substance from an ancillary reservoir or to a recovery process in the nervous transmission system, or to a mixture of some or all of these processes. The type of mixture will depend upon the relative velocities of the various processes. In the work to be described some results have been obtained which throw a little light on this problem.

Briefly, the state of the retina, after exposure to light, is measured by the course of recovery of its sensitivity after the stimulus has been discontinued. This mode of test reveals differences which are not easily or certainly detected while the eye is still exposed to the stimulus, possibly because it is a test more simply connected with the state of the retina than are tests carried out during the exposure of the light stimulus.

A few theoretical considerations may be introduced here in order to lead up to the subsequent discussion of the experimental results. The fact that the sensitivity of the eye is reduced by exposure to light and recovers in the dark shows that the photochemical decomposition by light of retinal substances is accompanied by a reverse reaction. No direct method is at present available to determine the nature and kinetics of the reverse reaction, except in very general terms, but a qualitative description of the phenomena to be expected can be deduced by assuming, for instance, that the reverse reaction is mono-molecular. Similar conclusions are reached by assuming other types of reverse reaction, but the mathematics is simplest for the monomolecular case. It should be emphasized that the theoretical treatment below is purely illustrative and is given for the purpose of crystallizing ideas on the subject. From the researches of Lythgoe, Goodeve and others on visual purple it appears probable that the reverse reaction of the photo-process in the retina is complicated, possibly a chain reaction, and representation by a mono-molecular reaction formula is only useful for qualitative illustration.

If n is the concentration of molecules of the photosensitive substance at time t , N is the equilibrium concentration in the dark and I is the intensity of light falling on the retina, the equation representing the kinetics of the reaction during exposure of the eye to light is

$$-\frac{dn}{dt} = KIn - L(N - n),$$

where K and L are constants of the forward and reverse reactions. Integrating this, and putting $n = N$ when $t = 0$ (initial state of dark adaptation),

$$n = \frac{N}{KI + L} (KIe^{-(KI+L)t} + L).$$

When the light is cut off the reverse reaction will proceed alone according to the equation

$$\frac{dn}{dt} = L(N - n),$$

which, on integration, gives

$$N - n = (N_0 - n_0)e^{-Lt},$$

where n_0 is the value of n at the moment of cutting off the light.

It remains to make some assumption which will connect concentration of photosensitive substance n with the threshold brightness sensitivity I_T of the eye. The simplest assumption is probably that a certain minimum

message must be sent to the brain in order that the test field may be perceived. It is also assumed that the equation relating to perception of the test field is of the simplified form

$$-\frac{dn}{dt} = KI_T n,$$

and that the message sent to the brain is a function of dn/dt . At the threshold, then, dn/dt is a constant and I_T is inversely proportional to n .

It is of particular interest to see what happens when the product It is constant, that is, when the eye is given a constant exposure in the photographic sense, the individual quantities I and t having various values. If the reverse reaction were absent or even very slow in comparison with the forward reaction, then it is obvious that the reciprocity law would be obeyed. If, however, the reverse reaction is comparable in speed with the

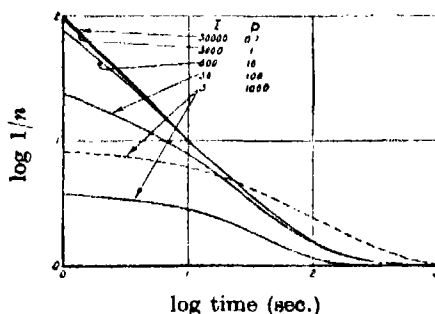


FIGURE 1. Theoretical recovery curves.

forward reaction, the state of affairs will be very different, and the curves of figure 1 have been calculated to exemplify what happens. K and L have each been taken equal to 10^{-2} , the product It to 3000, and N to unity. These values of K and L were chosen to give curves as similar as possible to the experimental curves.

The result, shown by the full-line curves in figure 1, is a family of curves in a fan-like formation, each curve starting at a different initial value and tending to the same final value at infinite time. It may also be noted that for small values of time of exposure (p) the curves tend to coalesce.

Another point of interest is what occurs if the value of L decreases when p becomes large. It is possible that the primary photochemical decomposition is followed by secondary chemical reactions of relatively slow speed. When p is large these secondary reactions might produce appreciable effects and the result would be represented approximately by a decrease

in the value of L . The dotted curve in figure 1 exhibits one case of the effect of a decrease in L ; a characteristic crossing over now occurs in the family of curves.

2. PLAN OF EXPERIMENTS

The experiments were mainly directed towards finding within what limits the reciprocity law holds for retinal photo-processes, as a step in the attempt to describe the behaviour of the eye by a limited number of general physical laws. The experiments were therefore grouped so as to bring together all those relating to a given total exposure, and each group carried through in as short a time as possible: a single day sufficed in most cases. An individual experiment was carried out by first giving the eye a period of dark adaptation, half an hour for foveal, an hour for parafoveal observations. The mode of experiment then varied according to whether foveal or parafoveal vision was used.

For foveal vision the eye was exposed to the primary conditioning stimulus for the requisite period and the time interval determined between cutting off the conditioning stimulus and first seeing the test field. This process was repeated for a series of intensities of the test field, giving a series of times, increasing as the test-field intensity decreased, from which the complete recovery curve could be plotted. The number of repetitions required for a complete curve could, in practice, be reduced to a half or a third by arranging a filter to be dropped into the test-field beam as soon as the test field was sighted, thus preparing for a longer time of recovery without the necessity for a fresh conditioning exposure.

In the case of parafoveal vision a single exposure to the primary conditioning stimulus was made and threshold measurements made thereafter, at first as quickly as possible, making use of the dropping filter technique if necessary, then at greater leisure as time proceeded. In this way a fairly complete recovery curve was obtained with a single conditioning exposure. The detail of the earlier parts of the curve is not so well shown as by the foveal method, but this is not of great importance, as the main interest for present purposes lies in the intermediate and final portions of the curve.

3. APPARATUS

The apparatus is shown in diagrammatic plan in figure 2. Both conditioning and test fields are seen by Maxwellian view, so that the effects of variation in pupil size may be avoided. The conditioning field beam is concentrated to a focus twice before reaching the eye in order to form

suitable points for the insertion of shutters, S_1 and S_2 . S_1 is a rapid-acting electromagnetic shutter working on the principle of the string galvanometer, the shutter blade being attached to the centre of the string; this serves for the control of all periods of exposure down to 0.03 sec. For shorter exposures, in order to obtain a reasonably high shutter efficiency, a dropping shutter S_2 is used, periods being calculated from distance of fall and width of opening. Neutral filters F_1 and F_2 are placed near the light source I , which is mounted in a light tight box, and neutral wedges W_1 and W_2 near points where the light beams are brought to a focus. For this reason the test-field beam is also concentrated to a focus once before reaching the eye. Auxiliary neutral filters in an automatic dropping mechanism are placed at F_3 . The shutter S_3 in the test-field beam is hand operated, and is used for parafoveal work to give exposures of approximately a second; the exact value is not at all critical when it is as long as this.

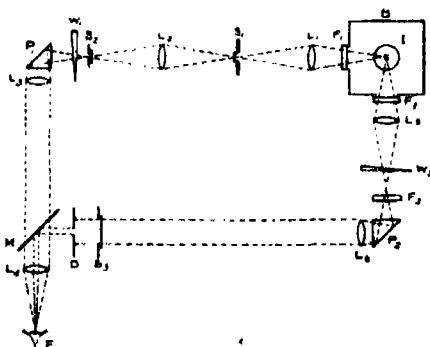


FIGURE 2. Diagrammatic plan of apparatus. I , lamp with small V-filament; B , light tight box; $L_1, L_2, L_3, L_4, L_5, L_6$, lenses; F_1, F_2, F_3 , neutral filters; W_1, W_2 , neutral wedges; S_1 , rapid acting electromagnetic shutter; S_2 , drop shutter; S_3 , hand operated shutter; P_1, P_2 , reflecting prisms; D , test-field diaphragm; M , pellicle mirror; E , eye of subject.

The test field is limited in size to a diameter of 0.5° by the diaphragm D . For the conditioning field the full aperture of the lens is used, giving a field diameter of 12° . The semi-reflecting mirror M , which enables the eye to see the test field superposed on the conditioning field, is a thin film of celluloid cemented to a framework of brass; when dry the shrinkage produces sufficient tension to make the film flat.

Field intensities are assessed by measuring the candle-power of the final image at the eye. This is done by comparison with a small lamp of known candle-power mounted beside the image at the same distance from the

photometer. The latter is an illuminometer with Maxwellian view of the external field. An image of the light source to be measured is formed in the eye ring of the instrument, so that the photometer field is seen full of light. In order to avoid errors due to the pupil efficiency effect the eye ring of the photometer is not larger than 0.5 mm. diameter. The apparent brightness of the visual field will be equal to the candle-power of the image at the eye divided by the area of the pupil in square feet. A pupil diameter of 3 mm. has been chosen as a convenient value.

4. RESULTS

Results have been obtained for both foveal and parafoveal vision over a wide range of intensities and times of exposure. All relate to the author's right eye and were obtained for white light in both conditioning and test fields, the colour temperature being approximately that of the gas-filled lamp. Positions in the visual field are specified by reference to the external field as seen from the observing position.

4.1 *Results for a foveal test area*

To investigate the foveal area of the retina the test field was centrally fixated as accurately as possible, and as it subtended an angle of 0.5° at the eye it fell completely within the retinal area usually accepted as foveal. The results are shown in figures 3-10, each figure containing a group of recovery curves for which the product of brightness of conditioning field (B) and period of exposure (p) is constant. The logarithm of the brightness threshold is plotted against the logarithm of the time after cutting off the conditioning stimulus, and the curves are labelled with their individual values of B and p .

These groups of curves can be compared with the theoretical curves of figure 1. Take, for instance, the curves of figure 7, for which the product Bp is nearly the same as for the theoretical curves. It is obvious that a numerical fit has not been obtained and hence, as expected, the assumptions made regarding types of reactions, etc., are too simple. Nevertheless, there is a definite family resemblance between the curves of figure 1 and those of figure 7. The points of resemblance may be enumerated: (a) the different starting points corresponding to different times of exposure; (b) the final convergence to a common value; (c) the tendency to coalesce for small values of p ; (d) the crossing over of curves for large values of p , pointing to some sort of secondary chemical reaction, or, possibly, diffusion of the end-products of the initial reaction. All these points of resemblance

may be observed also in figures 4-6. In figure 3 the crossing over of curves is absent, presumably because none of the times of exposure is very long. In figures 8-10 cross-over is also absent, but the explanation is probably different. It seems more likely that in these cases the total exposure, and hence the amount of photochemical decomposition, is so large that the tendency towards crossing over has merely brought the curves together over their lower ranges.

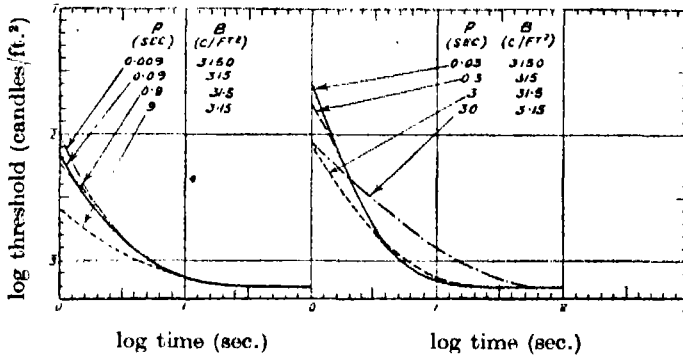


FIGURE 3. $pB = 28$.

FIGURE 4. $pB = 95$.

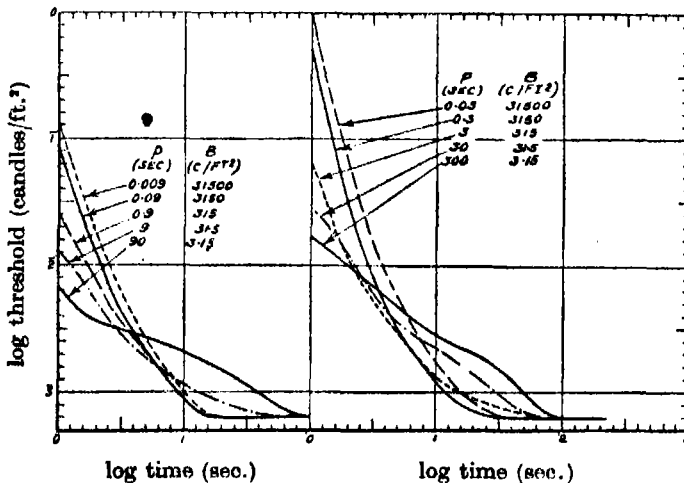


FIGURE 5. $pB = 280$.

FIGURE 6. $pB = 950$.

FIGURES 3-6. Variation of threshold with time.

4.2. Results for a parafoveal test area

Results for a parafoveal test area 8° to the nasal side of the fixation point are shown in figures 11-18. They are arranged in groups on the same plan as the foveal results.

In the parafovea there are, according to ideas now universally accepted, two main types of visual mechanism, photopic and scotopic, the former being present alone in the fovea in most subjects. It is to be expected, therefore, that the parafoveal recovery curves will show evidence of these

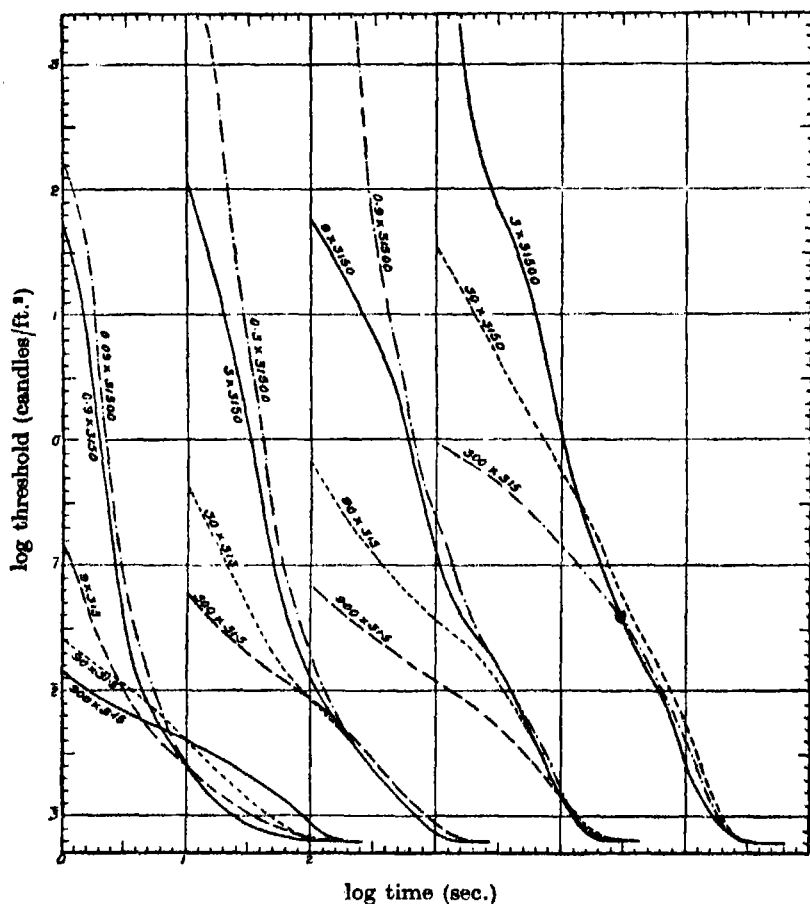


FIGURE 7. FIGURE 8. FIGURE 9. FIGURE 10.
 $pB = 2800$. $pB = 9500$. $pB = 2800$. $pB = 95,000$.

FIGURES 7-10. Variation of threshold with time.

two mechanisms. On looking through the parafoveal curves it will be seen that in all groups, apart from one or two special curves, there is an initial period in which the curves are in the fan formation already noted in the fovea, followed by a period of coincidence lasting until dark adaptation is complete. It is reasonable to assume that the initial period is showing the

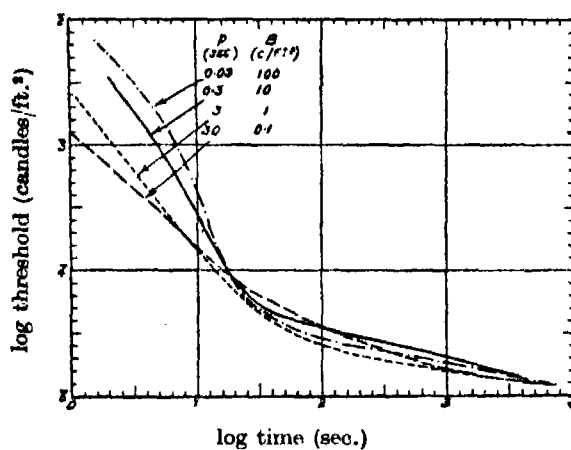


FIGURE 11

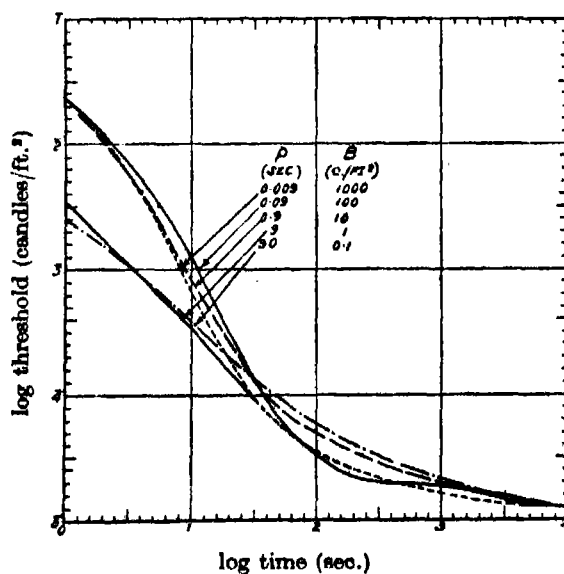


FIGURE 12

FIGURES 11-19. Variation of threshold with time. Parafovea 8°.

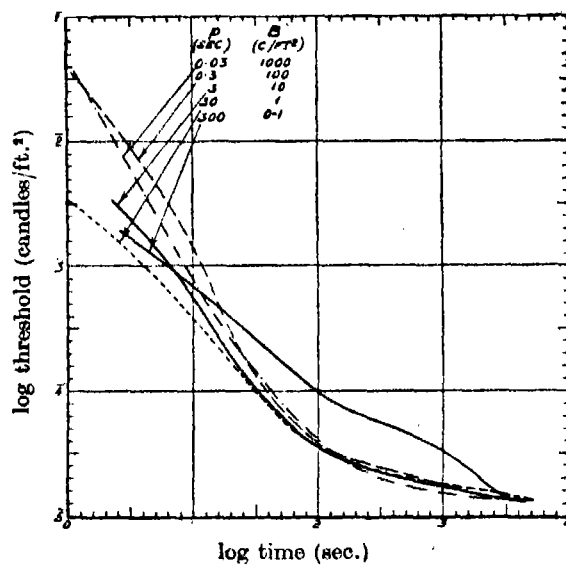


FIGURE 13

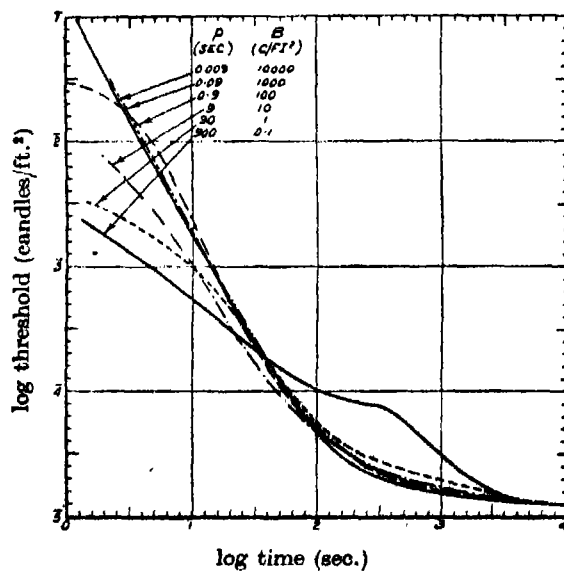


FIGURE 14

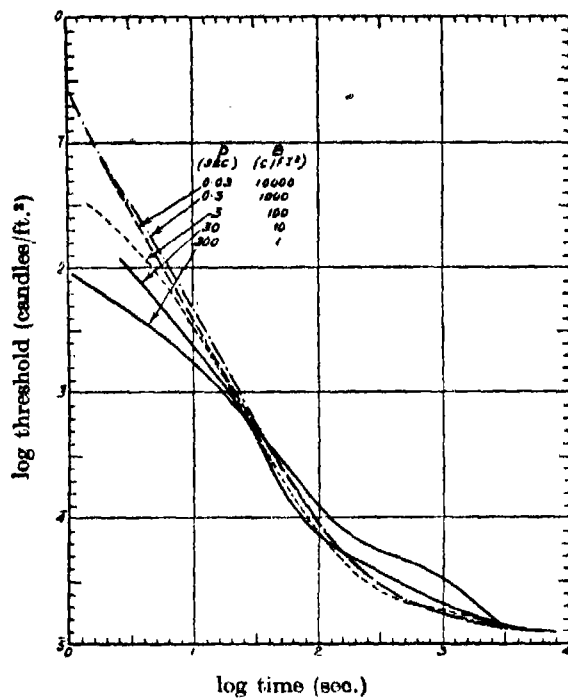


FIGURE 15

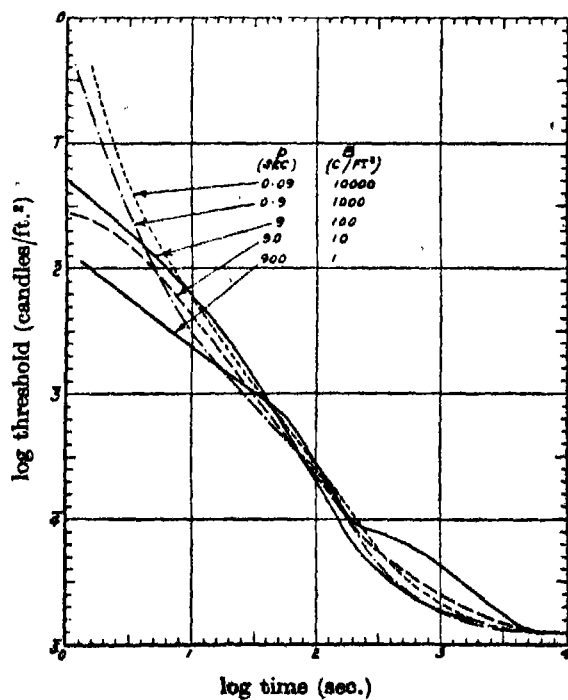


FIGURE 16

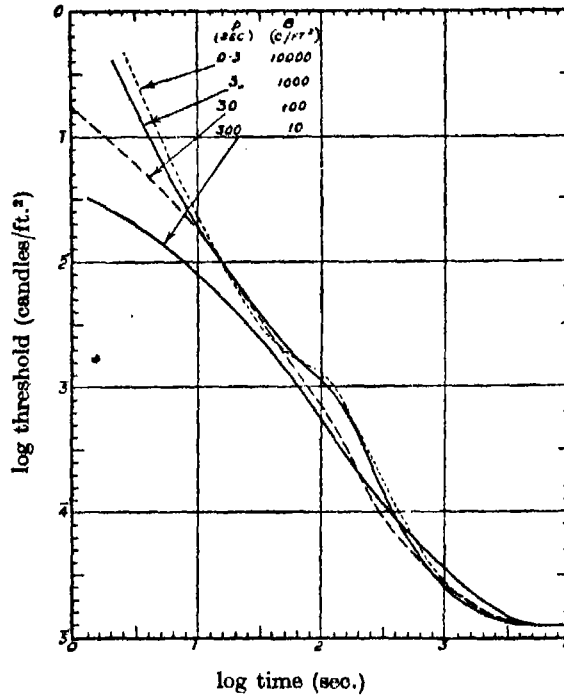


FIGURE 17

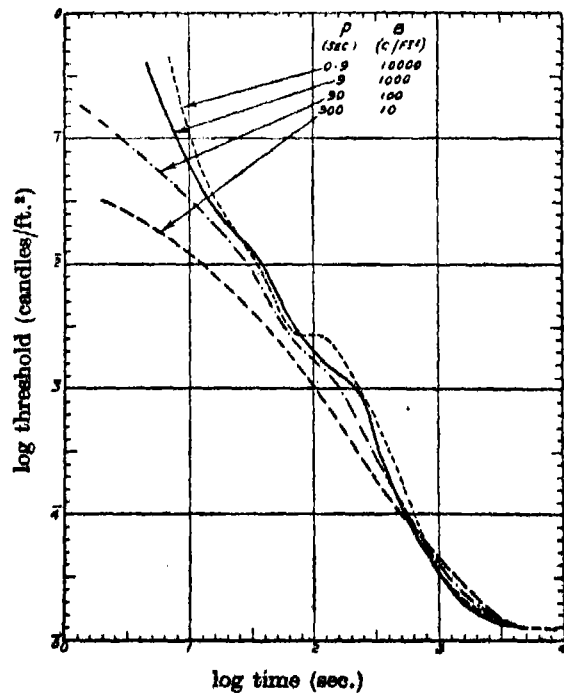


FIGURE 18

phenomena of photopic vision, the later period those of scotopic vision. The coincidence of curves in the later period is interpreted as showing that the reverse reaction is so slow compared with the forward reaction that the reciprocity law holds over a wide range of conditions to a fairly high degree of precision.

It may next be noted that in figures 13-16 the curve in each group corresponding to the longest periods of exposure of the conditioning field crosses over the other curves. The presumption is that here, as in the fovea, a secondary reaction is making itself felt. In figures 17 and 18 the cross-over effect is very small, not much greater than the probable error of the measurements. The explanation of this, as in the foveal case, is probably that the amount of photochemical decomposition is so high that the secondary reaction has very little effect in displacing the end point.

Finally, the curves of figures 17 and 18 exhibit a feature not clearly present in any of the other curves, namely, an apparent splitting-up into two portions, one above and the other below a value of log threshold of approximately 3.2. This again looks like a separation of the photopic and scotopic mechanisms. Such separation evidently requires a large total conditioning exposure and is favoured by a high intensity of conditioning stimulus.

5. GENERAL INFERENCES

The most important practical result obtained is that within certain limits the parafoveal visual process obeys the reciprocity law, enabling behaviour under large numbers of conditions to be predicted on the basis of relatively few experimental data. Explicitly, the predictable behaviour includes recovery of dark adaptation in fovea or parafovea when the initial conditioning stimulus is of short duration (under 1 sec. approximately), and the middle and later stages of recovery in the parafovea when the initial conditioning stimulus does not exceed 100 sec. in duration.

On the theoretical side it has been found that the phenomena of changing state of adaptation of the retina may be completely explained, in a qualitative way, by the fundamental principles of photochemistry, including a simple assumption of some type of secondary reaction following the main photochemical change. It may be noted that it has not so far been found necessary to introduce any component of recovery due to the nervous transmission to the brain. The quantitative explanation depends largely on the theory of the kinetics of chemical reactions, a subject in which theoretical finality does not appear to have been reached, so that finality in the visual problem is likewise unattained.

Further experiments on insect competition

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The growth of populations of the beetles *Tribolium confusum* and *Oryzaephilus surinamensis* was observed in media of wheat, coarse wholemeal flour and fine wholemeal flour, respectively. These were maintained at a constant level by the periodic transference of the insects to equal amounts of fresh media. Population growth was best observed in fine flour, from which all stages could be sifted out and counted. In populations of each species beginning with two adult males and two females, maxima for eggs, larvae and pupae succeeded each other and finally adults emerged and themselves rapidly rose to a maximum. The adult populations remained steady at the maximum, while egg, larval and pupal populations fluctuated round mean values. The rate of population growth was determined by the rates of oviposition and development on the one hand, and of cannibalism on the other. Such cannibalistic eating of eggs and, more important, of pupae by adults and larvae, rather than the limitation of food, also determined the maximum population size. A comparison of the rates of oviposition with the rates at which adults emerged showed that in such populations the mortality in the immature stages was over 99 %.

In competition with *Oryzaephilus*, which depended entirely upon mutual predation, *Tribolium* had the advantage because of its greater voracity. *Oryzaephilus* was driven out of the flour media which did not protect its pupae. But when the flour media contained glass tubing of such bore as to allow its larvae to enter it and pupate, but to exclude *Tribolium* adults and large larvae, *Oryzaephilus* survived together with *Tribolium* just as it did in wheat. In wheat, the beetle *Rhizopertha dominica* survived together with *Tribolium*. The three species *Rhizopertha*, *Tribolium* and *Oryzaephilus* also survived together in this medium. The results of these competitions support the contention that species with the same needs and habits are unable to survive together in the same environment while species which differ in needs or habits may do so.

The position at which equilibrium between any two of the competing species was reached was independent of the initial density of each species. When constants of the Lotka-Volterra simultaneous equations for the population growth of two species competing for the same limited environment were calculated from the experimental data, they led to inequalities corresponding to definite equilibrium positions for these equations. The actual equilibrium positions reached by the populations were in every experiment the same as those reached by the equations. The biological assumptions on which these equations are based are not, however, strictly true for *Tribolium* and *Oryzaephilus*.

Populations living in unrenewed flour rose to a maximum and then declined as the food became exhausted and 'conditioning' increased. As time passed, the age composition shifted from a majority of young stages to a majority of adults. The extinction of the *Tribolium* population was due to the failure of the larvae to develop and pupate, and of the *Oryzaephilus* population to this as well as to the cessation of oviposition. The adults, having failed to reproduce themselves, eventually died.

I. INTRODUCTION

It has been shown that the rate of population growth in 'unconditioned' flour of the beetle *Tribolium confusum* is determined on the one hand by the rates of oviposition and of development, and on the other by the rates of cannibalistic egg- and pupa-eating by larvae and adults (Chapman 1928; Park 1932, 1933; MacLagan 1932; Chapman & Baird 1934; Chapman & Whang 1934; Holdaway 1932; Stanley 1932 a, b, 1941). Beginning with a very unsaturated population, the rate of increase of the adult population will at first increase to a high value as more

females begin ovipositing, but will then fall off as the rate of oviposition decreases and the rate of cannibalism increases with increasing density. The adult population will cease to grow when eggs and pupae are found and eaten as fast as they are produced. Since the feeding stages of the beetle *Oryzaephilus surinamensis* also eat their own eggs and pupae, it is probable that the rate of population growth of this species too is determined by the factors just mentioned (Crombie 1943).

Tribolium is intrinsically superior (Smith 1929) to *Oryzaephilus* in competition since, first, it has under the same conditions a higher fecundity than the latter species. Secondly, the adults and larvae of *Tribolium* destroy *Oryzaephilus* eggs at a greater rate than adults of the latter species destroy the eggs of *Tribolium*. *Oryzaephilus* larvae do not attack *Tribolium* eggs. Also, the adults and larvae of *Tribolium* destroy *Oryzaephilus* pupae, while the feeding stages of the latter species do not attack *Tribolium* pupae. From this it has been deduced that when the two species are in competition *Tribolium* is likely to exterminate *Oryzaephilus* in environments which afford no protection to the eggs and pupae of the latter insect (Crombie 1943). With constant physical conditions, the intraspecific and interspecific factors controlling the population growth of these species were all density-dependent (Crombie 1942, 1943). This paper describes an experimental study, carried out during 1940-1, of the population growth of *Tribolium* and *Oryzaephilus* in various media when each species was either living alone or competing with the other species. A brief account is also given of the growth of populations of *Rhizopertha dominica* in competition with *Tribolium* and with both *Tribolium* and *Oryzaephilus*.

The populations were kept in a dark incubator at 30° C and 70 % R.H. Other technical precautions were similar to those described in a previous paper (Crombie 1942). All populations lived in standard environments of 10 g. of a food medium in jars of standard size (6.5 cm. diam. × 5.5 cm. high). Media of three different kinds were used: 'deeply cracked' wheat (as in Crombie 1945, § V), coarse wholemeal flour, and fine wholemeal flour. No instar of either *Tribolium* or *Oryzaephilus* is able to attack intact wheat grains. The coarse wholemeal flour was such that the majority of its large particles (wheat test) could just pass through a 1 mm. mesh. The fine flour was obtained as already described (Crombie 1943) by grinding up the coarse wholemeal flour and sieving it through no. 52 (extra quadruple) silk bolting cloth. This fine flour seems to have the same nutritional value as wheat because adults appeared in it as soon as they did in wheat (cf. tables 1, 4 and 5) (Park & Burrows 1942). The medium was renewed every week in some experiments, but not at all in others (see tables). The sex ratio of the original adults was always unity. The original females were young, and, to ensure copulation, had been confined before the experiment with two males each for 2 weeks. The fecundity of a number of these females was measured and for the original adults of populations which began with two or five such originals only those were used which had the following standard fecundities: *Tribolium* 9-11, *Oryzaephilus* 3-4, and *Rhizopertha* 6-8 eggs per female per day (Crombie 1942, 1943). This precaution was unnecessary in

populations which began with 100 original adults, since the average rates of oviposition of all such populations are likely to have been equal. The method of conducting the experiments was similar to that already described (Crombie 1945). All instars were counted in populations living in fine flour (§ III), but only adults could be counted in the other media. Censuses were made usually every 7 or 14 days. Each of the experimental jars was duplicated. Since the population growth in the duplicates was always very similar, each census value given in the figures and tables is the mean of the counts in the two duplicates.

At 30° C and 70 % R.H. the average duration of each *Tribolium* instar during development was as follows: egg, 6 days; larva, 17 days; pupa, 6 days; total, 29 days (emergence scatter about 5 days). There were usually six larval instars. The pre-oviposition period was 1-5 days (Brindley 1930; Stanley 1932a). Similar details for *Oryzaephilus* are given in Crombie (1943).

II. THE GROWTH OF POPULATIONS IN RENEWED WHEAT

(a) *Tribolium* (n_1) competing with *Oryzaephilus* (n_2). The growth of populations of each species alone in 'deeply cracked' wheat, beginning with four adults, is shown in table 1 (a, b) and figure 1. The *Tribolium* population had two periods of rapid increase separated by a period of slower increase. The *Oryzaephilus* population seems to have had a fairly uniform rate of increase until it approached the asymptote. A logistic equation fitted to the data in table 1 (b) is shown in a previous paper (Crombie 1945, figure 5b). The average longevity of the adults of each species was estimated as already described (Crombie 1945) from the number of dead adults found at each count, assuming the oldest to die first. The average longevity of *Tribolium* adults was 168 days, extremes being 50 days and something over 208 days. Pearl, Park & Miner (1941) found that the average longevity of this species at 28° C and 40-50 % R.H. was 178 days for males and 199 for females, extremes being from 0-5 to 540 days including both sexes. The average longevity of *Oryzaephilus* adults was 99 days, extremes being 79-137 days. The average rate of adult mortality in maximum populations of *Tribolium* was 0.0027 per adult per day, varying between 0.00017 and 0.0098. This value may be too low because of the habit of *Tribolium* of eating dead adults, which would make the values for longevity too high. In a maximum population natality is equal to mortality. In flour the fecundity of *Tribolium* at a density of 40 beetles per gram is approximately 2.5 eggs per adult per day (Crombie 1943). Assuming that fecundity has the same value in wheat, it follows that in maximum populations there is in the immature stages a mortality of about 99.8 % of the eggs laid. The average rate of adult mortality in maximum *Oryzaephilus* populations was 0.0034 per adult per day, varying between 0.00076 and 0.0057. At 40 beetles per gram the fecundity of this species would be approximately 0.7 egg per adult per day (Crombie 1942). About 99.5 % of the eggs laid must therefore have died in the immature stages, leaving only 0.5 % to produce new adults.

TABLE 1. THE GROWTH OF ADULT POPULATIONS OF *TRIBOLIUM* AND *ORYZAEPHILUS* IN WHEAT. MEDIUM RENEWED

days	(a)	(b)	(c)		(d)		(e)		(f)	
	Trib.	Oryz.	4 Trib. v. 4 Oryz.		100 Trib. v. 4 Oryz.		4 Trib. v. 100 Oryz.		4 Trib. v. 400 Oryz.	
	alone	alone	Trib.	Oryz.	Trib.	Oryz.	Trib.	Oryz.	Trib.	Oryz.
0	4	4	4	4	100	4	4	100	4	400
7	4	4	4	4	100	4	4	100	4	400
14	4	4	4	4	100	4	4	100	4	400
21	4	4	4	4	100	4	4	100	4	390
28	4	4	4	4	100	4	4	100	4	380
35	53	25	47	23	100	5	4	258	4	380
42	141	44	136	39	100	8	4	297	4	260
49	183	63	170	55	142	11	6	315	12	270
63	225	147	211	141	160	17	19	310	50	290
77	262	285	220	207	176	25	37	300	48	310
91	295	345	222	275	198	38	69	330	73	290
105	351	361	227	265	267	55	68	332	81	321
119	395	405	230	278	307	65	85	315	95	555
133	410	471	230	330	361	95	91	350	95	475
147	422	420	245	321	375	128	91	360	97	452
161	411	430	260	300	375	159	93	395	101	427
175	415	420	265	280	370	172	97	440	107	439
189	427	475	285	232	360	161	112	405	111	491
203	423	435	298	200	365	132	147	422	138	392
217	419	480	330	150	361	122	163	392	154	344
231	428	450	375	105	359	150	189	335	192	313
245	424	420	373	161	364	180	242	313	256	292
259	—	—	368	200	366	189	297	310	301	243
273	—	—	350	152	—	—	365	172	357	165
287	—	—	360	131	—	—	367	151	367	195
301	—	—	361	128	—	—	372	172	369	134
315	—	—	—	—	—	—	367	204	359	112
329	—	—	—	—	—	—	372	195	364	161

When the two species competed with equal initial numbers (table 1 (c)), each depressed the other but neither was exterminated. The *Tribolium* population reached a maximum of about 360 adults, while the *Oryzaephilus* adult population oscillated between 100 and 200. The *Oryzaephilus* population is thus much more depressed by the competition of *Tribolium* than it was by the competition of *Rhizopertha* or *Sitotroga* (Crombie 1945). This is to be expected, since the needs and habits of *Oryzaephilus* are much more like those of *Tribolium* than those of the other two species. When the initial populations of the two species were unequal (table 1 (d)–(f)), equilibrium between them was reached as before at adult populations of approximately 360 *Tribolium* and 100–200 *Oryzaephilus*. Now the adult populations cease to increase when eggs and pupae are destroyed as fast as they appear. In experiments (a) and (b) (table 1, figure 1), the populations remained more or less constant at the size then reached. In experiment (c) both species increased at

first with little or no competition at approximately the same rates as in (a) and (b), respectively. Population growth ceased temporarily when there were about 230 *Tribolium* and 300 *Oryzaephilus* adults. The adults of both species now began to die of old age, allowing more eggs and pupae to survive to adulthood and replace them. (The average longevity of the adults dying between the 119th and 245th days was 157 for *Tribolium* and 107 for *Oryzaephilus*.) But because of the greater

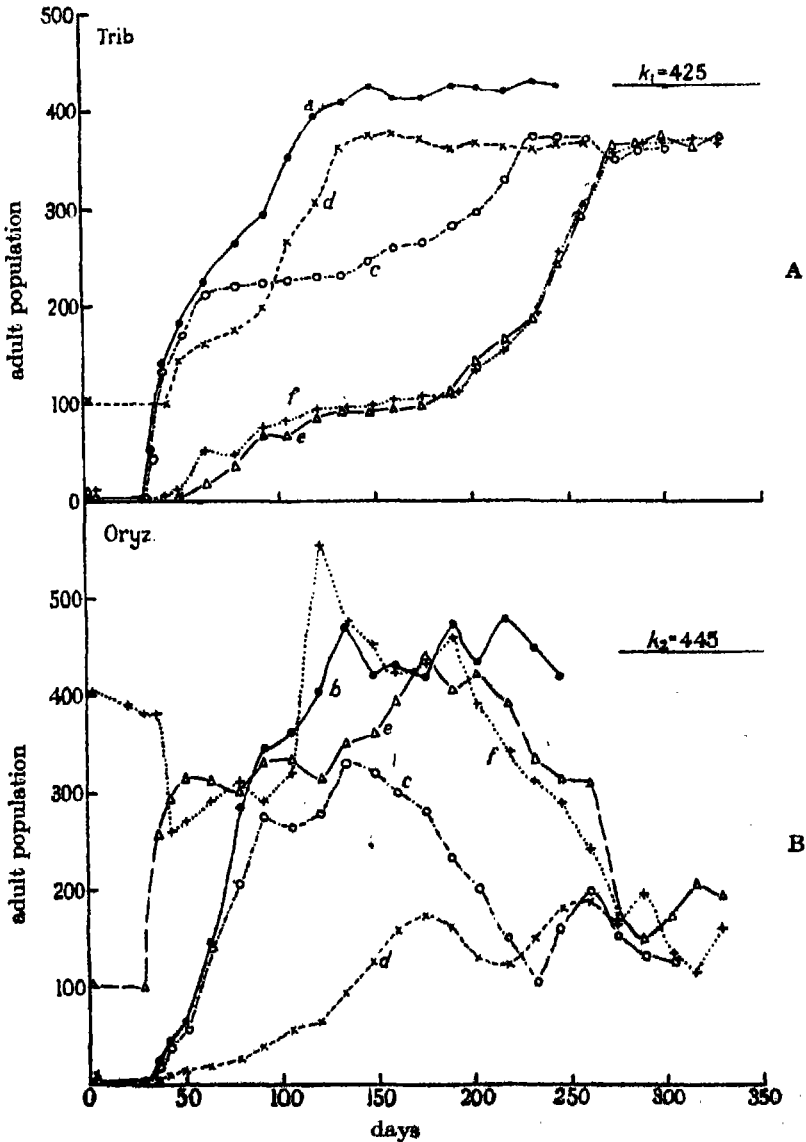


FIG. 1. The growth of adult populations of (A) *Tribolium* (n_1) and (B) *Oryzaephilus* (n_2), alone and in competition with each other, in renewed wheat. The curves (a-f) correspond to the data shown in table 1 (a-f).

rate of predation of its feeding stages *Tribolium* is intrinsically superior to *Oryzaephilus*. A larger proportion of the new adults emerging therefore belonged to the former species. The rate of emergence of *Tribolium* adults exceeded the adult death-rate, and its adult population increased. The rate of emergence of *Oryzaephilus* adults was lower than the adult death-rate of this species, and its adult population decreased. But the *Oryzaephilus* were evidently able to survive in part of the environment, for its adult population eventually oscillated between 100 and 200. These adults were able to prevent the *Tribolium* adult population rising above about 360. The *Oryzaephilus* survived because the larvae pupate inside hollowed-out wheat grains, to which they are attached by the anal end, and are protected from the predacious attacks of *Tribolium*. *Tribolium* pupae were nearly always found in the open. This will be discussed in detail later (§ III).

In experiments (c) and (f) the growth of the populations was similar to that in (c). *Tribolium* population growth was at first inhibited by a large increase in *Oryzaephilus* adults, but when the latter began to die of old age *Tribolium* adults began to increase and replace those of its rival. The average longevity of the *Tribolium* adults which died between the 119th and 273rd day was 161 days in (e) and 175 days in (f). The average longevity of the *Oryzaephilus* adults dying in the same period was 92 days in (e) and 94 days in (f). When four *Oryzaephilus* adults were competing with 100 *Tribolium* adults (d) the equilibrium positions were the same as in (c), but the *Oryzaephilus* population took a longer time to increase to this value. The populations at which equilibrium between the two species is reached is thus independent of the initial adult populations (cf. Park, Gregg & Luthermann 1941).

(b) *Tribolium* (n_1) competing with *Rhizopertha* (n_2). The population growth of *Rhizopertha* living alone was the same in 'deeply cracked' wheat as that in 'cracked' wheat which has already been described (Crombie 1945). The maximum population reached in 10 g. of wheat was about 338 adults. When *Rhizopertha* was competing with *Tribolium* neither species was eliminated, but the *Rhizopertha* population was very depressed (table 2, figure 2). Whatever the initial concentrations of each species, equilibrium was always reached with about 420 *Tribolium* and thirty-five *Rhizopertha* adults. The ecological niches for which the two species compete are different in that the *Rhizopertha* larvae develop inside the grains while all *Tribolium* stages are found outside them. As the niches of *Rhizopertha* and *Tribolium* are theoretically more different than those of *Tribolium* and *Oryzaephilus*, it is surprising that *Rhizopertha* should prove more sensitive to the competition of *Tribolium* than *Oryzaephilus* does. The reason for this may be that while *Oryzaephilus* attacks the eggs and pupae of *Tribolium*, *Rhizopertha* eats none of the stages of any species (Crombie 1942). *Rhizopertha* would thus compete with *Tribolium* only for food, whereas *Tribolium* would destroy the eggs, pupae (if it found them) and possibly the larvae of *Rhizopertha*. Possibly the latter species survives at all only because its larvae bore into the grains for food and thus find protection for themselves and for their pupae.

TABLE 2. THE GROWTH OF ADULT POPULATIONS OF *RHIZOPERTHA* AND *TRIBOLIUM* IN WHEAT. MEDIUM RENEWED

days	(a) 100 <i>Rhiz.</i> v. 100 <i>Trib.</i>		(b) 200 <i>Rhiz.</i> v. 10 <i>Trib.</i>		(c) 50 <i>Rhiz.</i> v. 400 <i>Trib.</i>	
	<i>Rhiz.</i>	<i>Trib.</i>	<i>Rhiz.</i>	<i>Trib.</i>	<i>Rhiz.</i>	<i>Trib.</i>
0	100	100	200	10	50	400
14	100	95	200	10	47	400
28	100	82	171	10	33	390
42	98	74	140	91	23	376
56	95	120	63	103	23	380
70	84	131	51	154	23	371
84	55	145	40	185	22	360
98	51	210	37	230	27	387
112	41	284	20	270	31	425
126	37	317	25	303	33	421
140	39	322	28	339	30	421
154	41	375	33	382	32	411
168	30	390	29	402	33	419
182	35	422	35	411	—	—
196	35	429	32	409	—	—

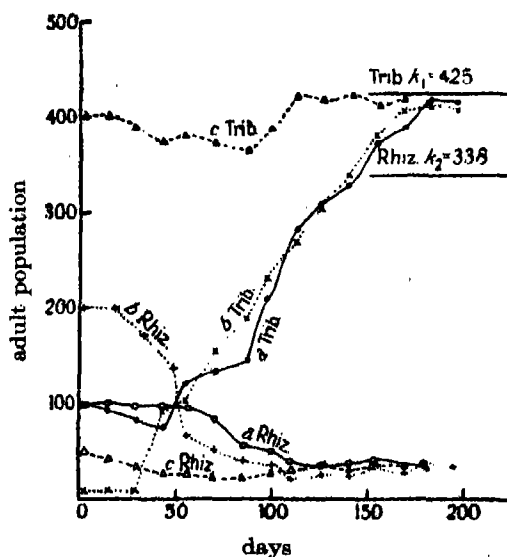


FIGURE 2. The growth of adult populations of *Tribolium* (n_1) and *Rhizopertha* (n_2) competing in renewed wheat. The curves (a-c) correspond to the data shown in table 2 (a-c). The horizontal straight lines represent the maximum populations (k_1 and k_2) reached by each species, respectively, when it was living alone.

(c) *Competition of Tribolium, Oryzaephilus and Rhizopertha.* The initial population consisted of 100 adults of each species (table 3, figure 3). After a period of fluctuation a position of equilibrium was reached. The *Oryzaephilus* and *Rhizopertha* populations become stabilized at approximately the same values as when

each of them competed only with *Tribolium* (tables 1 and 2). These two species compete very little with each other in this medium (Crombie 1945), so that the equilibrium sizes of their populations here probably depend mainly on the presence of *Tribolium*. The latter species is considerably more depressed here than when it competed with either species alone. It is impossible with the information available to explain these results completely. It may be noted that although neither *Tribolium* nor *Oryzaephilus* would be able to survive at all in intact wheat, they would be able to do so if the grains had been damaged by the larvae of *Rhizopertha* or by its adults emerging from pupae inside the wheat.

TABLE 3. THE GROWTH OF ADULT POPULATIONS OF *RHIZOPERTHA*, *TRIBOLIUM* AND *ORYZAEPHILUS* IN WHEAT. MEDIUM RENEWED

days	<i>Rhiz.</i>	<i>Trib.</i>	<i>Oryz.</i>	days	<i>Rhiz.</i>	<i>Trib.</i>	<i>Oryz.</i>
0	100	100	100	112	86	130	115
14	100	83	100	126	77	138	125
28	85	75	100	140	64	149	180
42	59	68	115	154	63	166	225
56	103	123	100	168	58	180	185
70	133	135	110	182	55	184	187
84	130	140	115	196	54	192	185
98	121	133	115	210	55 ✓	191	186

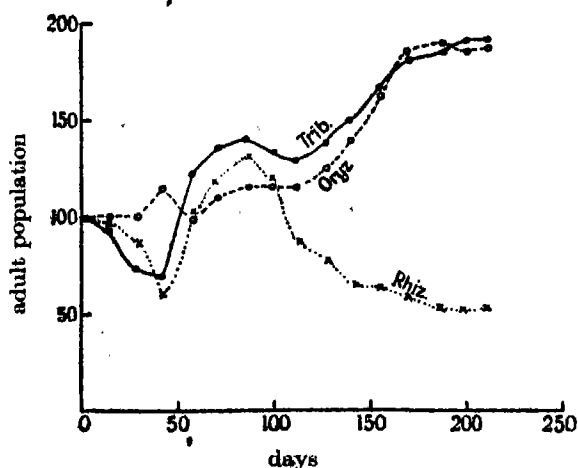


FIGURE 3. The growth of adult populations of *Tribolium*, *Oryzaephilus* and *Rhizopertha* competing in renewed wheat (table 3).

III. THE GROWTH OF POPULATIONS IN RENEWED WHOLEMEAL FLOUR

(a) *Tribolium* competing with *Oryzaephilus* in fine flour. The method of making the counts of populations growing in this medium was similar to that used by Chapman (1928) and Park (1934). The flour was sieved and all instars were counted at intervals of approximately 7 or 14 days. The contents of the jars were first

gently shaken by hand through a coarse sieve which retained adults, pupae and larger larvae, while flour, smaller larvae and eggs passed through. The insects retained by the coarse sieve were then gently removed and counted. Care must be taken here, since in *Tribolium* vigorous shaking lowers fecundity (Park 1934). The material passing through the sieve was put into a mechanical shaker and sieved through no. 52 (extra quadruple) silk bolting cloth, which allowed only the flour to pass through. This flour was discarded. All stages had now been separated from the flour and were easily counted under a binocular microscope. Dead insects were removed. The medium in each of the jars was now renewed with 10 g. of fresh fine flour, the eggs and small larvae, after they had been counted, being first thoroughly mixed up with the fresh flour. This fresh flour plus eggs and small larvae, together with the adults, pupae and large larvae, was now replaced in the jar concerned, and returned to the incubator. It was impossible to estimate accurately the longevity of *Tribolium* adults in this medium, since many of the dead adults were eaten by their living descendants.

TABLE 4. THE POPULATION GROWTH OF *TRIBOLIUM*
ALONE IN FINE FLOUR. MEDIUM RENEWED

days	eggs	larvae	pupae	adults	total
0	—	—	—	4	4
7	87	15	—	4	106
14	12	67	—	4	83
21	10	98	3	4	115
28	28	63	70	12	173
35	52	40	61	45	198
42	73	3	33	90	199
49	140	7	10	130	287
65	160	24	2	144	320
77	250	11	14	141	406
91	125	13	5	136	279
106	175	7	8	129	319
125	225	10	4	141	380
141	148	7	3	151	309
153	285	4	0	140	429
172	180	6	1	135	322
186	150	13	3	131	297
201	190	7	2	132	331
215	165	13	5	123	306
235	100	31	8	126	265
250	165	15	3	133	316

The course of population growth of *Tribolium* (table 4, figure 4) is similar to that described by Chapman (1928) and Stanley (1932 a, b). There was an initial accumulation of eggs which went on until the hatching out of the larvae. The first egg maximum occurred on day 7. Then, as the number of larvae increased, the egg population decreased, because some became larvae and others were eaten by these larvae (Chapman 1933; Chapman & Whang 1934; Crombie 1943). The larval

population now increased to a maximum on day 21, then decreased when larvae first began to pupate. Corresponding to this decrease in the number of feeding larvae there was now an increase in the egg population. The pupal population rose to a maximum on day 28 and decreased when the adults began to emerge. When the adults of the first filial generation began to oviposit, the egg population underwent a second increase to reach a maximum on day 49. A second larval maximum occurred on day 65, and a second pupal maximum on day 77. On day 49 the adult population reached a maximum of approximately 135 and remained at that size. The egg population eventually came to fluctuate about a mean of approximately 160, and the larval and pupal populations underwent extremely damped fluctuations at a low level. The final maximum population including all stages was approximately 320, or 32 insects per gram.

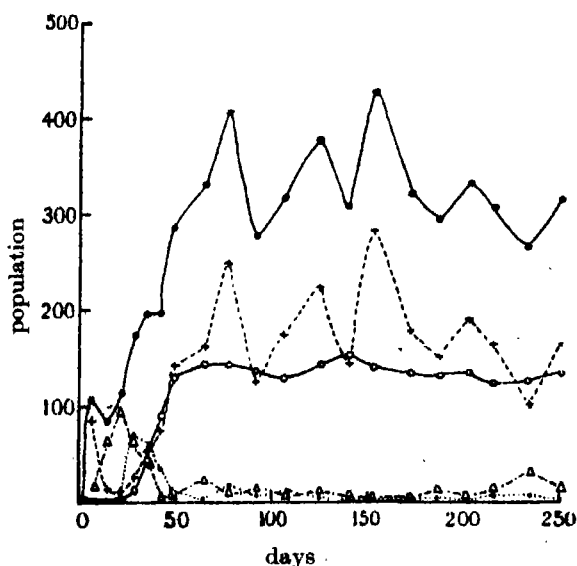


FIGURE 4. The population growth of *Tribolium* alone in renewed fine flour (table 4).

Eggs +---+; larvae \triangle --- \triangle ; pupae; adults \bigcirc — \bigcirc ; total \bullet — \bullet .

The changes which occurred during the population growth of *Oryzaephilus* alone were similar to those just described for *Tribolium* (table 5 and figure 5). The rate of increase of the total *Oryzaephilus* population was less than that of *Tribolium*, probably because of the greater fecundity of the latter species (Crombie 1943). The first egg maximum occurred on day 7 and was followed by a first larval maximum on day 14 and a first pupal maximum on day 28. The first new adults appeared on day 28. The adult population now increased to reach a maximum of approximately 120 by the 141st day. The egg, larval and pupal populations also increased and came to fluctuate about mean values of approximately 100, 175 and 40 respectively. The final maximum population including all stages was approximately 440, or 44 insects per gram. The proportion of larvae and pupae in this

population is much greater than that in the *Tribolium* population, possibly because *Oryzaephilus* is much less voracious than the latter species (table 11). The maximum adult populations of both species are much lower in fine flour than in crushed wheat.

TABLE 5. THE POPULATION GROWTH OF *ORYZAEPHILUS*
ALONE IN FINE FLOUR. MEDIUM RENEWED

days	eggs	larvae	pupae	adults	total
0	—	—	—	4	4
7	29	28	—	4	52
14	19	36	—	4	59
21	14	29	13	4	60
28	19	26	46	14	105
35	34	21	20	20	95
42	119	48	15	32	214
49	98	118	16	36	268
65	121	109	62	50	342
77	128	125	103	53	409
91	68	120	47	68	303
107	65	85	18	82	250
125	53	95	20	91	259
141	113	150	35	118	416
153	85	165	45	116	411
172	150	190	38	116	494
186	105	160	22	113	400
201	95	170	50	118	433
215	75	185	73	122	455
235	60	200	30	110	400
250	80	175	60	125	440

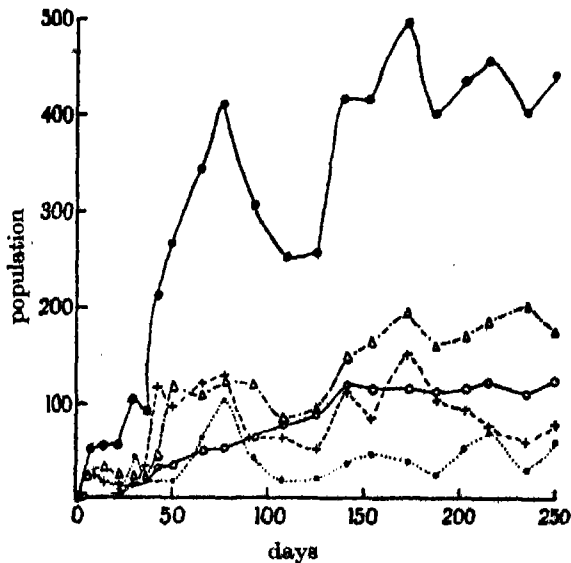


FIGURE 5. The population growth of *Oryzaephilus* alone in renewed fine flour (table 5).

Eggs +---+; larvae Δ---Δ; pupae•; adults ○—○; total ●—●.

In the next experiment the initial population consisted of four young adults (sex ratio unity) of each species (table 6 and figure 6). The *Tribolium* population rose as before to a maximum of approximately 320 (all stages), while the *Oryzaephilus*, after an initial rise, were eventually driven out. At first the population growth of both species was much the same as when each was living alone. Egg, larval and pupal maximum followed each other. The pupal maximum of *Tribolium* was then followed by an increase in the number of adults, which reached a maximum of approximately the same value as that in table 4. But there was practically no increase in *Oryzaephilus* adults. The total *Oryzaephilus* population reached a maximum of fifty-four on day 7. In table 5 the total population rises to 105 on day 28. The absence of such a rise in table 6 must have been due to the *Tribolium* larvae, since they were the only feeding stage of that species yet present. The instar most visibly affected by these larvae is the pupa. In table 5 there are forty-six pupae present on day 28 and in table 6 only eleven (§ III (b)). Eggs are also eaten by *Tribolium* larvae. The *Oryzaephilus* total population began to decrease rapidly from day 35, the numbers of all instars falling to low values. This decrease in *Oryzaephilus* coincided with a rapid increase in the numbers of *Tribolium* adults. As this seems to be the only new factor appearing in the system at this time, the disappearance of the *Oryzaephilus* must have been mainly due to the eating of its eggs and pupae by *Tribolium* adults, although the larvae must also have played their part. The *Oryzaephilus* adults eventually died of old age, leaving *Tribolium*

TABLE 6. THE POPULATION GROWTH OF *TRIBOLIUM* VERSUS
ORYZAEPHILUS IN FINE FLOUR. MEDIUM RENEWED

days	<i>Tribolium</i>					<i>Oryzaephilus</i>				
	eggs	larvae	pupae	adults	total	eggs	larvae	pupae	adults	total
0	—	—	—	4	4	—	—	—	4	4
7	88	12	—	4	104	25	25	—	4	54
14	45	58	—	4	107	17	30	—	4	51
21	18	111	3	4	136	11	32	8	4	55
28	8	77	63	8	156	9	30	11	4	54
35	22	34	44	65	165	3	19	3	4	29
42	25	17	38	92	170	9	12	—	5	26
49	175	16	9	118	218	—	10	—	5	15
65	150	10	18	118	296	—	3	—	5	8
77	160	7	3	117	287	—	1	—	5	6
91	175	14	3	119	311	2	2	—	4	8
106	135	12	3	112	262	—	1	—	4	5
125	210	15	5	119	349	—	—	—	2	2
141	158	20	6	125	309	—	—	—	1	1
153	195	11	3	115	324	—	—	—	1	1
172	210	17	3	115	345	1	—	—	1	2
186	85	22	12	108	227	—	—	—	1	1
201	135	6	8	116	265	—	—	—	1	1
215	110	19	3	119	251	1	—	—	1	2
235	190	17	8	115	330	—	—	—	1	1
250	120	21	7	113	261	—	—	—	0	0

in sole occupation of the environment. As already mentioned (§ I), this result was predicted in a previous paper from the intrinsic natures of the two species (Crombie 1943). *Tribolium* evidently has two advantages over *Oryzaephilus*. First, its fecundity is much greater. At the densities reached the fecundities of both species would be practically unaffected by density (Crombie 1942). Over the first 7 days here the average fecundities (in terms of eggs and larvae counted; some would have been eaten) of *Tribolium* and *Oryzaephilus* were 7.2 and 3.1 eggs per female-day, respectively. Secondly, the differential rates of egg- and pupae-eating of the two species are much in favour of *Tribolium* (Crombie 1943). Not only are the *Oryzaephilus* eggs and pupae destroyed at a greater rate, but *Tribolium* pupae are completely immune from the attacks of all stages of *Oryzaephilus*. This is perhaps the deciding factor, since all *Tribolium* eggs which hatch are henceforth immune from attack by *Oryzaephilus*, while the pupae of the latter species are readily attacked by *Tribolium*.

When the initial population consisted of four *Oryzaephilus* adults (sex ratio unity) and 100 *Tribolium* adult males, the latter were sufficient to prevent the formation of any new *Oryzaephilus* adults. The females of the latter species oviposited and about ten eggs and four larvae were found at each count (the experiment was continued for 98 days), but no pupa was ever found. This proves that in the experiment shown in table 6 the newly emerged *Tribolium* adults could have exterminated the *Oryzaephilus* from day 35 onwards, without the assistance of larval predation.

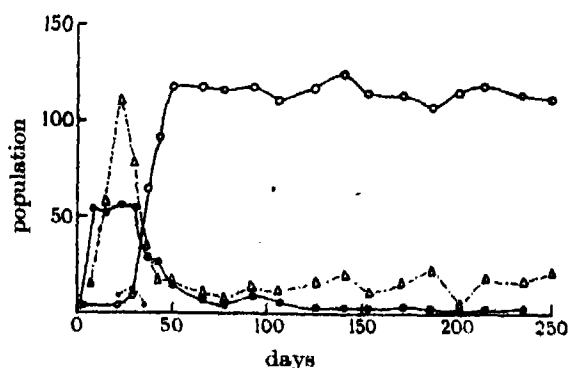


FIGURE 6. The population growth of *Tribolium* and *Oryzaephilus* competing in renewed fine flour (table 6).

Tribolium larvae Δ---Δ; adults ○—○; *Oryzaephilus* pupae ●.....●; total ●—●.

When the initial population consisted of four *Tribolium* and 100 *Oryzaephilus* adults, the *Oryzaephilus* population increased enormously at first (table 7). But in spite of this the rate of increase of the *Tribolium* population was almost as great as when no *Oryzaephilus* were present (table 4). As before, egg, larval and pupal maxima of both species succeeded each other. There was no increase in *Oryzaephilus* adults. Coinciding with the rapid increase in the *Tribolium* adult population

there was a decline in the total *Oryzaephilus* population. The total *Tribolium* population eventually came to reach a maximum of approximately 320, while the *Oryzaephilus* gradually disappeared as the adults of this species died of old age. The average longevity of the *Oryzaephilus* adults was 122 (39-246) days. This was not significantly different from that in wheat (§ II (a)). The total maximum *Tribolium* populations in tables 4, 6 and 7 are approximately equal, but the relative proportions of adults and larvae are different (table 11). Possibly there is a sort of dynamic equilibrium between the two feeding stages (cf. Chapman 1933; Chapman & Whang 1934).

TABLE 7. THE GROWTH OF POPULATIONS WITH INITIALLY FOUR *TRIBOLIUM* AND 100 *ORYZAEPHILUS* IN FINE FLOUR. MEDIUM RENEWED

days	<i>Tribolium</i>					<i>Oryzaephilus</i>				
	eggs	larvae	pupae	adults	total	eggs	larvae	pupae	adults	total
0	—	—	—	4	4	—	—	—	100	100
7	80	20	—	4	104	300	100	—	100	500
14	110	50	—	4	164	200	200	—	100	500
25	10	102	17	5	134	60	325	5	100	490
32	80	50	70	15	215	150	320	5	100	575
39	115	5	51	88	259	290	350	7	97	744
52	50	85	0	134	269	145	280	14	85	524
67	125	18	55	135	333	75	65	2	80	222
86	95	15	4	155	269	25	23	1	60	109
101	110	5	2	163	280	15	6	—	55	76
113	140	4	1	170	315	5	1	—	43	49
132	175	2	0	163	340	5	0	—	50	55
147	150	3	0	155	308	7	1	—	43	51
161	175	2	1	150	328	4	—	—	38	42
176	160	1	0	160	321	0	—	—	30	30
196	160	0	1	163	324	0	—	—	25	25
211	165	3	0	162	330	0	—	—	16	16
231	145	6	0	161	312	2	—	—	16	18
246	150	10	0	155	315	—	—	—	11	11

Now *Oryzaephilus* adults feed mostly in the surface layers of the flour. The larvae, which feed anywhere in this medium, do not destroy *Tribolium* eggs. The flour in the standard jars used in the above experiments was approximately 2 cm. deep, so that all *Tribolium* eggs in the medium except those in the surface layers would escape, and, as already pointed out, once it has hatched a *Tribolium* is immune from attack by *Oryzaephilus*. An experiment was therefore performed in which the initial population of 100 *Oryzaephilus* adults and four *Tribolium* adults were placed in 10 g. of fine flour in a large dish so that the depth of the flour was not more than approximately 0.2 cm. No detailed results of this experiment will be published because although the rate of increase of the *Tribolium* population was at first depressed by the presence of the *Oryzaephilus*, the latter species was eventually driven out. The rate of increase of the *Tribolium* over the first 7 days was only 3.2 eggs and larvae per female-day, as compared with 7.15 when the numerical

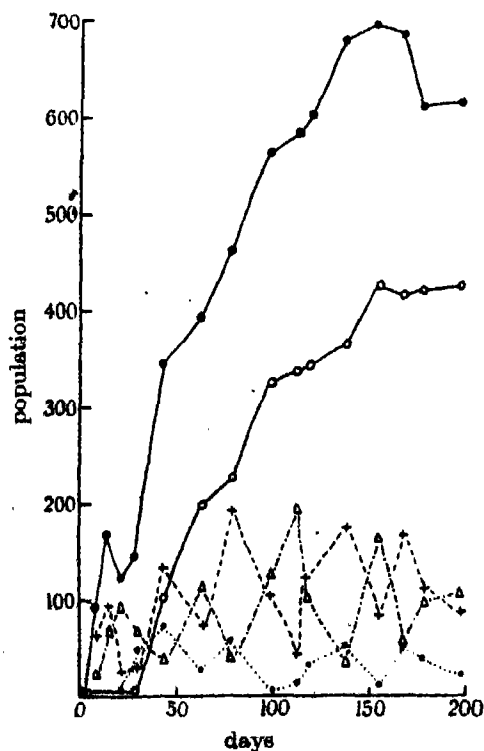
superiority of the *Oryzaephilus* was the same but the flour was deeper (table 7). The adults reached their maximum on day 133 instead of day 52, but otherwise the pattern of events was much the same as in table 7. With 250 *Oryzaephilus* and four *Tribolium* adults in this medium, the rate of increase of *Tribolium* over the first 7 days was only reduced to three. Although the *Oryzaephilus* total population reached a maximum of 1111 on day 56 when the *Tribolium* population was only fifty-seven, the latter species reached its maximum of approximately 320 (135 adults) by day 133 and the last *Oryzaephilus* adult died of old age on day 340.

(b) *Tribolium* competing with *Oryzaephilus* in fine flour with protection for eggs and pupae. In wheat the *Oryzaephilus* were not exterminated by the *Tribolium*, but in fine flour they were. The following experiments were performed to analyse the difference between these two media as they affected the competition between the two species. Thirty sections of glass tubing 2 cm. long were added to each 10 g. of fine flour. In some jars the tubing had a bore of 1 mm. Now the approximate widths of different instars of *Tribolium* at the widest part were as follows: adult 1.1 mm.; last instar larva 1.1 mm.; pupa 1.15 mm. (cf. Brindley 1930). For *Oryzaephilus* the same measurements were as follows: adult 0.6–0.8 mm.; last instar larva 0.5 mm. (Back & Cotton 1926); pupa 0.8–0.9 mm. Therefore all instars of *Oryzaephilus* could enter the 1 mm. tubing, while adults and large larvae of *Tribolium* could not enter this, but could enter 2 mm. tubing. All instars could be counted in this medium, as insects inside the tubing could be readily seen under a binocular microscope. The age composition (table 11) of the populations of *Tribolium* shown in table 8 and of *Oryzaephilus* shown in table 9 are probably not unlike the age composition of the populations of these species in wheat (table 1).

Consider first the competition of the two species in flour with 2 mm. tubing (table 8). The population growth of *Tribolium* alone (figure 7) was very similar to that shown in table 8. *Oryzaephilus* had practically no effect upon the growth of *Tribolium*. The adult population of the latter species rose to a maximum of approximately 410, and the total population to a maximum of approximately 650. The adult maximum was approximately equal to that in wheat (table 1). By protecting the eggs and pupae from the feeding stages and reducing cannibalism, the 2 mm. tubing thus allows a fuller use of the food resources of the fine flour. Almost all the pupae were found inside the tubing, and the pupal population remained at a higher level than in fine flour without tubing (table 4). The larval population was much higher than in table 4, which suggests that a higher proportion of the eggs also escaped destruction (cf. table 11). The egg population was approximately the same as in table 4. These results agree with those of Holdaway (1932) and of Stanley (1934, 1941). The former author found that the maximum adult populations reached in flour under different conditions of atmospheric moisture were lower with lower relative humidities. Assuming that the population ceased to increase when eggs and the pupae were eaten as fast as they appeared, he attributed this result to these instars being eaten at a greater rate at lower humidities, because then the water content of the flour was too low to satisfy the requirements of the

TABLE 8. THE POPULATION GROWTH OF *TRIBOLIUM* VERSUS *ORYZAEAPHILUS* IN FINE FLOUR WITH 2 MM. BORE GLASS TUBING. MEDIUM RENEWED

days	<i>Tribolium</i>					<i>Oryzaephilus</i>				
	eggs	larvae	pupae	adults	total	eggs	larvae	pupae	adults	total
0	—	—	—	4	4	—	—	—	4	4
7	67	19	—	4	90	17	9	—	4	30
14	87	72	—	4	163	22	16	—	4	42
21	25	91	5	4	125	12	34	2	4	52
28	20	64	40	6	130	8	30	6	6	50
42	127	32	67	89	315	9	27	8	10	54
63	60	94	12	182	348	10	20	2	13	45
77	170	40	50	202	462	7	8	—	9	24
98	120	120	10	325	575	6	4	—	6	16
112	80	170	10	325	585	1	—	—	7	8
119	100	125	55	315	595	2	—	—	4	6
140	200	45	45	345	635	—	—	—	5	5
154	70	190	8	410	678	—	—	—	5	5
168	150	65	50	415	680	1	—	—	5	6
182	135	62	32	415	644	—	—	—	4	4
196	80	120	12	412	624	—	—	—	1	1

FIGURE 7. The population growth of *Tribolium* alone in renewed fine flour with 2 mm. bore glass tubing.Eggs +---+; larvae Δ --- Δ ; pupae; adults \circ — \circ ; total \bullet — \bullet .

feeding stages. MacLagan (1941) has criticized this view and suggested that at the higher humidity the maximum population was higher because the larval period was shorter and fecundity and longevity greater (cf. Bushnell 1938; Park & Burrows 1942). But it seems that as long as the maximum possible rate of canni-

TABLE 9. THE POPULATION GROWTH OF *ORYZAEPHILUS* ALONE IN FINE FLOUR WITH 1 MM. BORE GLASS TUBING. MEDIUM RENEWED

days	eggs	larvae	pupae	adults	total
0	—	—	—	4	4
7	28	30	—	4	62
14	22	48	—	4	74
21	18	30	14	4	66
28	40	35	48	14	137
42	180	50	59	40	329
56	150	310	120	85	665
77	190	230	82	127	629
89	230	260	85	250	825
103	200	270	73	361	904
127	170	270	110	390	940
141	110	310	87	419	926
155	120	560	64	413	1157
169	170	300	86	381	937
183	120	360	41	407	928

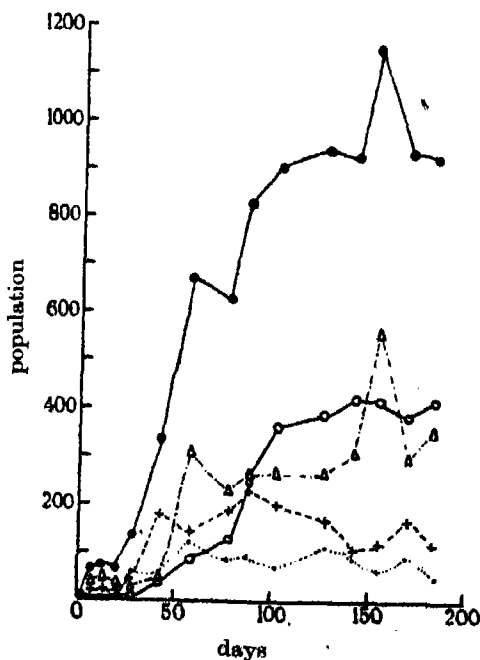


FIGURE 8. The population growth of *Oryzaephilus* alone in renewed fine flour with 1 mm. bore glass tubing (table 9).

Eggs +---+; larvae \triangle --- \triangle ; pupae; adults \circ — \circ ; total \bullet — \bullet .

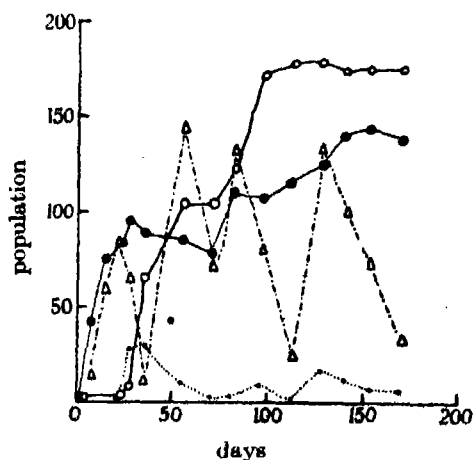
balism is higher than the rate at which eggs and pupae are produced, the maximum population will be determined by cannibalism alone. Furthermore, cannibalism can have been the only factor involved in the experiments described above (table 8). Stanley (1941) found the maximum possible rate of adult egg-eating to be greater than the fecundity (about 8 eggs per female per day) of his *Tribolium*. Stanley (1934) also found that the number of eggs present in a culture at any time T between the start of the culture and the time of hatching of the first egg was decreased when the flour was less nutritious, less palatable or less tightly packed, and furthermore, that if hatching were prevented (Stanley 1941), the asymptotic value to which the egg population moved was similarly affected. He suggested that where the flour was less nutritious or less palatable the relative preference for eggs as opposed to flour as food increased. This result does not necessarily conflict with that of Park (1935), who found that the rate of egg-eating in 'conditioned' flour was lower than that in fresh flour, since 'conditioned' media may have a definitely 'poisoning' effect (Crombie 1942) besides being merely unpalatable.

Two-millimetre tubing protected *Oryzaephilus* eggs and pupae from its own feeding stages, for when this species was living alone it reached a maximum population of approximately 200 adults and approximately 500 total. The pupae were almost always found inside the tubing. But the protection offered was apparently incomplete, since the maximum population reached was lower than that in wheat (table 1) or with 1 mm. tubing (table 9). No details will be given because the factors involved are better illustrated by population growth in the latter medium. Two-millimetre tubing, into which *Tribolium* larvae and adults could crawl, did not protect the *Oryzaephilus* from its rival (table 8). The total *Oryzaephilus* population was first inhibited by the *Tribolium* larvae on day 28 (cf. tables 9, 10), and began to decrease rapidly with the rise of the *Tribolium* adult population from day 42 onwards. The remaining *Oryzaephilus* adult females continued to oviposit, but all their offspring were eaten in the immature stages by *Tribolium* and all the adults eventually died of old age, leaving *Tribolium* in sole occupation of the medium.

With 1 mm. tubing (table 9, figure 8) the *Oryzaephilus* adult population rose to a maximum of about 400, or equal to that in wheat (table 1), while the total maximum population was about 930. The 1 mm. tubing thus protects *Oryzaephilus* eggs and larvae from its own feeding stages more efficiently than the 2 mm. tubing does. Practically all the pupae were found inside the tubing. The maximum *Tribolium* population was approximately the same whether living alone (control) in this medium or competing with *Oryzaephilus*. Therefore details of the latter only will be given (table 10, figure 9). The *Tribolium* adult population reached a maximum of only approximately 175, and the total population a maximum of approximately 420. This species could not pupate inside the 1 mm. tubing, and consequently the pupae were not protected as they were by the wide tubing. The pupal population was higher than in table 4, but lower than in table 8. The egg and larval populations, on the other hand, were approximately equal to those in table 8. This seems

TABLE 10. THE POPULATION GROWTH OF *TRIBOLIUM* VERSUS *ORYZAEPHILUS* IN FINE FLOUR WITH 1 MM. BORE GLASS TUBING. MEDIUM RENEWED

days	<i>Tribolium</i>					<i>Oryzaephilus</i>				
	eggs	larvae	pupae	adults	total	eggs	larvae	pupae	adults	total
0	—	—	—	4	4	—	—	—	4	4
7	65	14	—	4	83	30	8	—	4	42
14	80	60	—	4	144	38	32	—	4	74
21	12	85	5	4	106	28	50	2	4	84
28	10	65	25	8	108	22	38	27	9	96
35	135	10	20	65	230	16	30	30	13	89
56	60	146	10	104	320	13	19	10	44	86
70	150	73	28	105	356	17	15	2	44	78
77	170	133	29	122	454	50	23	2	35	110
98	150	80	16	173	419	33	26	9	40	108
112	195	24	21	179	419	55	5	1	54	115
126	70	135	7	179	391	30	15	17	64	126
140	85	100	7	175	367	30	24	11	76	141
154	172	72	4	174	422	37	19	6	82	144
168	210	34	6	175	425	41	4	5	89	139

FIGURE 9. The population growth of *Tribolium* competing with *Oryzaephilus* in renewed fine flour with 1 mm. bore glass tubing (table 10).

Tribolium larvae Δ --- Δ ; adults \circ — \circ ; *Oryzaephilus* pupae•; total \bullet — \bullet .

to suggest that the most important factor limiting the adult population of *Tribolium* in fine flour is the cannibalistic eating of pupae. The 1 mm. tubing offers the same protection to the eggs and larvae as does the 2 mm. tubing, but the latter alone is able to protect the pupae from the adults, the only stage which eats them (Crombie 1943). Consequently when 2 mm. tubing was present the maximum adult population was higher than otherwise. In table 4 the number of pupae is very low in the saturated population, while the number of eggs is high, which also

suggests that population size is limited by the destruction of pupae rather than of eggs (Voûte 1937). Since 2 mm. tubing protected *Tribolium* pupae from *Tribolium* adults, it seems probable that it also protected *Oryzaephilus* pupae from them. One of the chief reasons why *Oryzaephilus* was unable to survive in competition with *Tribolium* with 2 mm. tubing must therefore have been that its pupae were destroyed by large *Tribolium* larvae (Crombie 1943). The latter enter the tubing before pupation. This conclusion is supported by the observation that on day 28, before the great increase of *Tribolium* adults, there are forty-eight *Oryzaephilus* pupae present in table 9 and twenty-seven in table 10, but only six in table 8. The 1 mm. tubing effectively protected the *Oryzaephilus* pupae from *Tribolium* adults and large larvae (figure 9). The maximum population of *Oryzaephilus* was about eighty adults, and 140 including all stages. Both species continued to exist together.

TABLE 11. APPROXIMATE AVERAGE PERCENTAGE AGE COMPOSITION IN SATURATED (MAXIMUM) POPULATIONS OF *TRIBOLIUM* AND *ORYZAEPHILUS* UNDER DIFFERENT CIRCUMSTANCES. (THESE VALUES FLUCTUATE IN TIME)

table	eggs %	larvae %	pupae %	adults %
<i>Tribolium</i>				
4	54.7	3.4	1.1	40.8
6	53.7	5.1	1.8	39.4
7	48.2	1.1	0.2	50.5
8	17.9	17.2	4.4	60.5
10	36.3	20.0	3.2	40.5
<i>Oryzaephilus</i>				
5	21.5	40.4	10.1	28.0
9	13.2	38.8	7.0	41.0
10	25.4	11.0	5.4	58.2

(c) *Tribolium* competing with *Oryzaephilus* in coarse wholemeal flour. Detailed results need not be given. The population growth of each species alone (beginning with four adults) was practically the same as that in wheat. *Tribolium* reached a maximum adult population of approximately 420 and *Oryzaephilus* of approximately 400. The coarse wholemeal flour thus seems to have protected the eggs and pupae of each species from their own feeding stages as effectively as wheat does. *Oryzaephilus* larvae build out of the coarse particles of this medium a cocoon in which they pupate. But they were not sufficiently protected from *Tribolium* to survive competition with it. When the initial population consisted of four adults of each species the *Oryzaephilus* adult population rose to twenty-three (when the *Tribolium* adult population had reached 113), then gradually declined and died out, while the *Tribolium* adult population rose to about 420. Now the lower the rate of cannibalism the higher is the level of population of either predator or prey stages

or both, at which the prey stages are eaten as fast as they appear and population growth ceases. If the rate of cannibalism was lower in coarse wholemeal flour than in fine flour, then this would explain the higher adult maxima at which population growth ceased (cf. Holdaway 1932; Stanley 1934, 1941). But unless the coarse wholemeal flour provides absolute protection from *Tribolium* for the *Oryzaephilus* pupae, as 1 mm. tubing does, any difference in the relative rates at which the two species destroyed each other would result in the extermination of that with the lower rate of destructive activity, just as it would in fine flour (Crombie 1943). The only difference is that the rate of extermination would be slower in coarse wholemeal flour, where the rate of predation is lower. Coarse wholemeal flour does not provide absolute protection for *Oryzaephilus* pupae. One hundred males and fifty large larvae of *Tribolium* destroyed in 3 days all of twenty *Oryzaephilus* pupae with which they were confined in 10 g. of this medium. This explains the extermination of *Oryzaephilus* by *Tribolium* in this medium, the lower rate of extermination being reflected by the higher maximum (23) reached by the *Oryzaephilus* adult population here as compared with that (5) in fine flour (table 6). The addition of either 1 or 2 mm. tubing to the medium did not affect the rate of growth or the maximum population reached by either species alone. But, as in fine flour, *Oryzaephilus* was able to survive competition with *Tribolium* with 1 mm. tubing, although not with 2 mm. tubing. With 1 mm. tubing the *Oryzaephilus* adult population reached a maximum of approximately ninety and remained there.

IV. THE GROWTH OF POPULATIONS OF *TRIBOLIUM* AND *ORYZAEPHILUS* IN UNRENEWED FINE WHOLEMEAL FLOUR

Ten grams of fine flour were placed in each jar at the beginning of the experiment, and the growth and decline of populations in this limited amount of food observed in the usual way. The old flour was replaced after each census had been taken. The population growth of *Tribolium* alone is shown in table 12, and that of *Oryzaephilus* alone in table 13. A population which initially consisted of four adults of each species was also observed, but no details will be given as the *Oryzaephilus* were soon exterminated, and the *Tribolium* population followed a course similar to that shown in table 12. When living alone, each species survived for over 600 days, then died out. As the populations grew and declined, the age composition changed. The percentage of each instar present after different periods is shown in brackets beside the corresponding census figure. The growing populations were characterized by a high proportion of young stages, while in the declining populations the adults predominated. Such changes in age composition with the growth and decline of populations have also been observed in man (Carr-Saunders 1936, chapter x), bees (Bodenheimer 1937*a, b*), and in natural populations of *Collembola* and *Acarina* (Ford 1938). The depression of the relative rate of increase of the growing population, so that the adult population reached a maximum, was in both species probably mainly due to cannibalism, as it was in renewed media. But the times taken

for the adults to reach these maxima were longer, and the maxima reached were lower, than in the latter media (tables 4, 5). These results agree with those of Park (1938a) and Park, Miller & Lutherman (1939), who observed that conditioned media decreased the rate of larval development of *Tribolium*. The decline of the populations from the maxima was presumably brought about by the exhaustion of the food supply and the conditioning of the medium. The media gradually began to lose weight from about the 200th day. After the adults had died out, only about 2 g. remained of both the *Tribolium* and the *Oryzaephilus* media, and this was brownish grey and sour-smelling. These substances were used in the experiments described in a previous paper (Crombie 1943) to test the effect of conditioned media upon fecundity, and as mentioned there young adult females are capable of ovipositing in it.

TABLE 12. THE POPULATION GROWTH OF *TRIBOLIUM* ALONE
IN FINE FLOUR. MEDIUM NOT RENEWED

days	eggs	larvae	pupae	adults	total
0	—	—	—	4	4
7	84 (86.6 %)	9 (9.3 %)	—	4 (4.1 %)	97
14	76 (64.4 %)	38 (32.2 %)	—	4 (3.4 %)	118
21	38	66	—	4	108
28	39 (28.6 %)	85 (62.6 %)	8 (5.9 %)	4 (2.9 %)	136
35	34 (25.6 %)	52 (39.1 %)	35 (26.3 %)	12 (9.0 %)	133
42	52	56	30	44	182
48	50 (27 %)	43 (23 %)	27 (14.5 %)	66 (35.5 %)	186
65	53	34	12	106	205
77	79	20	5	110	214
91	45	38	4	113	200
106	35 (18.8 %)	38 (20.5 %)	1 (0.5 %)	112 (60.2 %)	186
125	13	53	1	126	193
141	14	38	3	112	167
153	25 (14 %)	40 (22.4 %)	1 (0.5 %)	113 (63.1 %)	179
172	16	30	1	110	157
186	19	25	2	101	147
201	20 (13.4 %)	24 (16 %)	0	106 (70.6 %)	150
215	15	27	1	106	149
235	30	28	0	109	167
250	30 (18.4 %)	25 (15.4 %)	0	108 (66.2 %)	163
283	25	6	0	107	138
312	37 (25.5 %)	10 (6.9 %)	0	98 (67.6 %)	145
326	34	7	2	96	139
340	52	12	0	90	154
391	55 (34 %)	13 (8.4 %)	2 (1.2 %)	92 (56.4 %)	162
433	25	15	2	91	133
545	4 (7 %)	5 (8.8 %)	1 (1.8 %)	47 (82.3 %)	57
593	0	1	0	20	21
639	0	0	0	5 (100 %)	5

The number of eggs present in the *Tribolium* population (table 12) remained high until day 433, but at the next census (day 545) was greatly reduced, and eventually

oviposition ceased altogether. By this time the adults had begun to die out. Except for two on day 391 no new adults (easily recognized by their light colour) were produced after day 283. All those present in the population on day 433 would thus be at least 150 days old, and the average longevity of adults under more optimum conditions than here was 168 days (§ II (a)). These ancient adults were black and sluggish. By day 545 they would therefore all be dying of old age, and it is interesting that the egg population remained high until this occurred. The oviposition period is probably almost as long as the life of the adult females (Good 1933; Park 1934). Park (1938*b*) has observed the same phenomenon. Now larvae and pupae continued to appear until nearly all the adults had died out, but Park (1938*a*) and Park *et al.* (1939) have shown that the conditioning of the medium by larvae or adults increases the larval and pupal mortality and the duration of the larval period of *Tribolium*. The final decline of the population must therefore have been due to the failure of the immature stages to develop and particularly their failure to metamorphose into adults, followed by the death of the existing adults from old age.

Holdaway (1933) observed that the starvation of newly hatched *Tribolium* larvae led to a reversal of sex and an excess of females. The proportion of males in 900 well-fed adults from three pairs of duplicate cultures in renewed flour (tables 4, 6 and 7) was 51 %, varying between 50.4 and 52 % in different populations. On day 391 the sex ratio of the 336 adults from two pairs of duplicates in unrenewed flour was unity (50 % of each sex), varying between 58 % males and 58 % females in different populations. There was no significant difference between the sex ratio of the populations in the two types of media. But it is possible that a reversal of sex may have occurred and was masked by other factors such as adult mortality. The sexes were distinguished by squeezing each adult until the tip of its genitalia appeared. This did not affect either their oviposition or their longevity.

The changes in the *Oryzaephilus* population were more marked than in those of *Tribolium*. No new adults were born after day 215, and the adult population began to die off from this point also. The egg and larval populations, however, fell drastically before this, from day 172 onwards. The fall in the larval population was accompanied by a rise in the number of pupae on day 172, presumably because of the decreased cannibalism, but the latter soon fell also. The dying out of the population of this species thus seems to have been due in the first place to the cessation of oviposition, while at the same time those eggs which were produced failed to develop into adults. The oviposition period of this species is said by Back & Cotton (1926) to be between a sixth and two-thirds of the length of life of the adult females. The existing adults eventually died of old age. The average longevity of the adults was 107 (47–172) days. This was not significantly different from that in renewed flour (§ III (a)). The 132 adults present in the two duplicate populations on day 215 were examined and the sex ratio found to be approximately unity, as it was in well-fed populations (table 5).

TABLE 13. THE POPULATION GROWTH OF *ORYZAEPHILUS* ALONE
IN FINE FLOUR. MEDIUM NOT RENEWED

days	eggs	larvae	pupae	adults	total
0	—	—	—	4	4
7	27 (75 %)	5 (14.5 %)	—	4 (10.5 %)	36
14	13	24	—	4	41
21	12 (16.6 %)	50 (69.5 %)	6 (8.3 %)	4 (5.6 %)	72
28	12	50	14	5	81
35	10	42	14	6	72
42	20 (25.3 %)	24 (30.3 %)	26 (33 %)	9 (11.4 %)	79
48	33	41	18	13	105
65	43	60	24	26	153
77	68 (33 %)	83 (40 %)	24 (11.6 %)	32 (15.4 %)	207
91	35	115	28	39	217
107	55	115	30	47	247
125	90 (23 %)	195 (50 %)	43 (11 %)	63 (16 %)	391
141	148	133	31	68	380
153	85	165	20	65	335
172	50 (16.7 %)	140 (46.7 %)	43 (14.3 %)	67 (22.3 %)	300
186	15	73	16	58	162
201	15	37	4	63	119
215	11 (10.2 %)	24 (22.5 %)	6 (5.6 %)	66 (61.7 %)	107
235	18	13	8	64	103
250	13 (15 %)	20 (23 %)	2 (2.3 %)	52 (59.7 %)	87
285	2	3	2	43	50
312	2 (4.7 %)	4 (9.3 %)	4 (9.3 %)	33 (76.7 %)	43
326	5	5	3	25	38
340	2	4	2	25	33
391	2	2	0	20	24
433	0	3 (15.8 %)	0	16 (84.2 %)	19
545	0	2	0	15	17
593	0	0	0	12 (100 %)	12
639	0	0	0	5	5

V. MATHEMATICAL MODELS ✓

The rate of population growth of *Tribolium* and *Oryzaephilus* and competition between them is determined almost entirely by the rates of oviposition and of development on the one hand, and by the rates of cannibalism and predation on the other. The effect of the limitation of food as such upon increase is negligible compared with these factors. The population growth of these species in fine flour may therefore be studied in the light of the direct observations of these processes made in this medium (Crombie 1943). The eating of eggs and pupae probably takes place after random encounters between feeding stages (adults and larvae) and prey stages (eggs and pupae). The relationship between these stages would then be analogous to a second order chemical reaction. Let g = number of eggs per gram at time t , g_0 = initial number of eggs per gram, and n = number of adults per gram. Then $-\frac{dg}{dt} = kn g$, therefore $\frac{1}{g} dg = -k n dt$, therefore $\log g = -k n t + c$.

When $t = 0$, $g = g_0$, therefore $C = \log g_0$, i.e. $\log g/g_0 = -knt$. When $t = 1$, $\log g/g_0 = -Kn$. With values of n from 1 to 20 a straight line may, owing to the experimental error, equally well be drawn through the points obtained when either $\log g/g_0$ or g/g_0 is plotted against n (Crombie 1943, text-figures 1* and 2). In the populations studied n never reaches 20 so that a linear relationship between the proportion of eggs surviving after unit time (g/g_0) and adult density (n) has been assumed. Similarly, a linear relationship has been assumed between the proportion of any prey stage surviving after unit time and the density of any feeding stage present. This assumption becomes incorrect, as it should have been pointed out, at the artificially high adult densities present in experiments described in a previous paper (Crombie 1943, pp. 81, 97). The maximum rates of egg-eating and of pupa-eating of which these insects are capable are of course higher than the rates of oviposition and of pupation, respectively. If it were not for hatching and ecdysis, prey stages would thus tend to accumulate until they were found and eaten as fast as they were produced (cf. Stanley 1941).

Let q represent the proportion of eggs eaten per day by one individual, h represent the hatching period in days, p represent the proportion of pupae eaten per day by one individual, and j represent the pupal period in days. Now in a population of one species, if there are N feeding individuals per gram of flour in the population, the proportion of eggs to survive 1 day will be $(1 - qN)$, since, at the values of N reached in these populations, the proportion eaten is linearly related to N , and the proportion to survive h days will be $(1 - qN)^h$. This is the proportion of eggs which will hatch into larvae, and if larval mortality be ignored as being small compared with that in the egg and pupal stages (cf. Park 1938a), this represents the proportion of eggs which become pupae. Similarly, if it be assumed that the percentage rate of pupa-eating is linearly related to the density of the eaters, the proportion of pupae to survive j days and thus emerge as adults is equal to $(1 - pN)^j$. In renewed media pupal mortality apart from that due to cannibalism is small (cf. Park 1938a). The proportion of eggs to become adults is therefore equal to $(1 - qN)^h (1 - pN)^j = A$, and the proportion destroyed is equal to $(1 - A)$ (cf. Stanley 1932-41). A has meaning of course only between 0 and 1.

When the two species are competing, the terms q_{11} and p_{11} must be introduced to represent the proportions of eggs and pupae, respectively, of the first species (N_1) eaten by itself; q_{12} and p_{12} to represent the proportions of the same eaten by the second species (N_2); q_{22} and p_{22} to represent the proportions of eggs and pupae of the second species eaten by itself; and q_{21} and p_{21} to represent the proportions of the same eaten by the first species. The proportion of eggs of the first species to become adults under these circumstances is equal to

$$(1 - q_{11}N_1)^{h_1} (1 - q_{12}N_2)^{h_1} (1 - p_{11}N_1)^{j_1} (1 - p_{12}N_2)^{j_1},$$

* In this figure the lettering on the right-hand ordinate should read '% eggs eaten per day' instead of '% eggs eaten per female-day'.

and the proportion of eggs of the second species to become adults is

$$(1 - q_{22}N_2)^{h_2}(1 - q_{21}N_1)^{h_2}(1 - p_{22}N_2)^{j_2}(1 - p_{21}N_1)^{j_2}.$$

Now let m_1 and m_2 represent the rates of adult mortality in terms of deaths per adult per day, and $e_1f_1(n_1 + n_2)$ and $e_2f_2(n_2 + n_1)$ represent the rates of egg laying per adult per day of each species, where n_1 and n_2 are the respective adult densities. Here e represents the average maximum rate of oviposition per adult per day. The rate of oviposition is a function $f(n)$ of density, and the adults of both species are considered as equivalent to each other in reducing the rate of oviposition of either (Crombie 1943). If they were not, it would simply be necessary to introduce a proportionality term. The rate of increase of the adults of one species is then given by the equation

$$\frac{dn}{dt} = n[ef(n)(1 - qN)^h(1 - pN)^j - m]. \quad (1)$$

Where the two species were competing, the respective rates of increase of their adults would be given by the equations

$$\left. \begin{aligned} \frac{dn_1}{dt} &= n_1[e_1f_1(n_1 + n_2)(1 - q_{11}N_1)^{h_1}(1 - q_{12}N_2)^{h_1}(1 - p_{11}N_1)^{j_1}(1 - p_{12}N_2)^{j_1} - m_1], \\ \frac{dn_2}{dt} &= n_2[e_2f_2(n_1 + n_2)(1 - q_{22}N_2)^{h_2}(1 - q_{21}N_1)^{h_2}(1 - p_{22}N_2)^{j_2}(1 - p_{21}N_1)^{j_2} - m_2]. \end{aligned} \right\} \quad (2)$$

Now let α represent the ratio of the inhibiting effect of one N_2 on N_1 to that of one N_1 on N_1 ; and β represent the ratio of the inhibiting effect of one N_1 on N_2 to that of one N_2 on N_2 . If m_1 and m_2 be ignored as insignificant compared to mortality in the immature stages, and also the effect of density on the rate of oviposition since here this too is a relatively small factor, then the values of α and β are as follows:

$$\alpha = \frac{1 - (1 - q_{12})^{h_1}(1 - p_{12})^{j_1}}{1 - (1 - q_{11})^{h_1}(1 - p_{11})^{j_1}} = \frac{H_{12}}{H_{11}}, \quad \beta = \frac{1 - (1 - q_{21})^{h_2}(1 - p_{21})^{j_2}}{1 - (1 - q_{22})^{h_2}(1 - p_{22})^{j_2}} = \frac{H_{21}}{H_{22}}.$$

H_{11} represents the proportion of eggs of the first species destroyed in the egg or pupal instar by one individual (N_1) of that species; H_{12} the proportion of eggs of the same species destroyed by one individual (N_2) of the second species; H_{22} the proportion of eggs of the second species destroyed by one individual of that species; and H_{21} the proportion of eggs of the second species destroyed by one individual of the first species.

Equations (1) and (2) are defective in that they provide no information about the larval populations, which are lumped together with the adults as 'feeding stages' (N). In order to calculate α and β it will have to be assumed that the ratio between adults and larvae is constant, which it is not in fact. Now if *Tribolium* (n_1) is the first species and *Oryzaephilus* (n_2) the second, then the following values are known: $h_1 = 6$, $h_2 = 5$, $j_1 = 6$, and $j_2 = 6$ (§ I and Crombie 1943, § I). At a

density of 1 adult per gram, *Oryzaephilus* adults would eat per day 1.4 % of the *Tribolium* eggs present (Crombie 1943, table IX). Hence $q_{12} = 0.014$. To calculate the other p and q values it will have to be assumed that the proportion of larvae to adults remains constant. Let it be assumed that the numbers of the different instars of each species present per gram are as follows: for *Tribolium* one adult, one 4th instar larva and one 6th instar larva; for *Oryzaephilus* one adult, one 1st instar larva and one 4th instar larva. For simplicity it will be considered that these combinations of adult and larvae are represented in the equations by N_1 and N_2 respectively. From the rates at which *Tribolium* adults and larvae eat their own eggs (Crombie 1943, table IX) it will be seen that the proportion of eggs left at the end of 1 day in 1 g. of flour containing the *Tribolium* feeding stages mentioned above will be $0.946 \times 0.992 \times 0.956 = 0.898$, and the proportion destroyed will then be $1 - 0.898 = 0.102 = q_{11}$. Similarly,

$$q_{12} = 0.014, \quad q_{22} = 1 - 0.984 \times 0.996 \times 0.984 = 1 - 0.964 = 0.036,$$

and
$$q_{21} = 1 - 0.956 \times 0.995 \times 0.962 = 1 - 0.915 = 0.085.$$

In the same way

$$p_{11} = 0.012, \quad p_{12} = 0, \quad p_{21} = 1 - 0.976 \times 0.984 = 1 - 0.96 = 0.04,$$

and
$$p_{22} = 0.004.$$

Then
$$\alpha = \frac{H_{12}}{H_{11}} = \frac{1 - (1 - 0.014)^6}{1 - (1 - 0.102)^6 (1 - 0.012)^6} = \frac{0.09}{0.49} = 0.018,$$

$$\beta = \frac{H_{21}}{H_{22}} = \frac{1 - (1 - 0.085)^6 (1 - 0.04)^6}{1 - (1 - 0.036)^6 (1 - 0.004)^6} = \frac{0.498}{0.187} = 2.7.$$

It has been shown (Crombie 1943, § IV) that if one of the two species has an advantage over the other when they are competing for the same ecological niche (e.g. fine flour), the stronger will exterminate the weaker and itself occupy the niche entirely. The first species (n_1) will survive when the following inequality is satisfied,

$$e_1(1 - H_{11})(1 - H_{12}) > e_2(1 - H_{22})(1 - H_{21}),$$

and the second (n_2) will survive when this inequality is reversed. If a certain proportion of the weaker species (n_2) is protected from the other in certain regions of the environment, then both may survive. This may be represented mathematically by substituting the terms $(1 - z_{21})q_{21}$ and $(1 - y_{21})p_{21}$ for q_{21} and p_{21} respectively, in equations (2), where z_{21} and y_{21} respectively represent the proportions of eggs and pupae of the second species which are protected from the attacks of the first. At 1 adult per gram of flour the rates of oviposition of *Tribolium* and *Oryzaephilus* will be approximately $e_1 = 5$ and $e_2 = 1.5$ eggs per adult per day, respectively (Crombie 1943, tables I and IV). Then

$$e_1(1 - H_{11})(1 - H_{12}) = 5(1 - 0.49)(1 - 0.09) = 2.3,$$

and
$$e_2(1 - H_{22})(1 - H_{21}) = 1.5(1 - 0.187)(1 - 0.498) = 0.61.$$

With this inequality the first species should drive out the second, which it did.

The limitations as a general expression for population growth, of the Verhulst-Pearl 'logistic' equation for the population growth of a single species of organism in a limited environment under constant physical conditions, have already been discussed (Crombie 1945). The Lotka-Volterra simultaneous equations for the population growth of two species competing for the same limited environment are also discussed in the same paper. The Lotka-Volterra equations may be written

$$\frac{dn_1}{dt} = b_1 n_1 \left(1 - \frac{n_1}{k_1} - \frac{\alpha n_2}{k_1} \right), \quad \frac{dn_2}{dt} = b_2 n_2 \left(1 - \frac{n_2}{k_2} - \frac{\beta n_1}{k_2} \right). \quad (3)$$

Here n_1 and n_2 represent the population densities of each species in the mixed population; b_1 and b_2 , respectively, their potential rates of increase; and k_1 and k_2 the maximum densities of each species when living separately. α and β are the 'competition coefficients'. While $1/k_1$ represents the proportion by which the potential increase per individual of the first species is inhibited by one individual of the same species, α/k_1 represents the proportion by which it is inhibited by one individual of the second species; and vice versa for $1/k_2$ and β/k_2 . The biological meaning of α and β here is precisely the same as that defined in connexion with equations (2). On integration there are shown to be four possible positions of equilibrium, when $dn_1/dt = dn_2/dt = 0$, corresponding to whether n_1 , n_2 or both species survive whatever their initial population densities, or whether either n_1 or n_2 survive and this depends chiefly on their initial population densities. The particular equilibrium position reached depends only upon the relative magnitudes of α , β , k_1 and k_2 , and is independent of initial population densities (see Crombie 1945).

As populations of *Tribolium* and *Oryzaephilus* are separated during growth into distinct generations, they will not increase strictly in geometric progression (cf. Stanley 1932a). Secondly, as seen from equations (1) and (2), neither intraspecific nor interspecific inhibition of increase is linearly related to population density. Therefore neither of the biological assumptions of equations (3), viz. that in the absence of checks increase is geometric and that all inhibiting factors are linearly related to population density, is strictly correct for these species under the conditions described. But separation of the generations may be eliminated either by considering only adult population growth (which is more or less continuous) or by measuring the growth of the total population from generation to generation. The divergence between the actual relationship between survival and density and the linear relationship assumed by equations (3) is no greater than the experimental error due to the variability of the rates of cannibalism and predation (Crombie 1943). The equations may be most easily studied at points where dn_1/dt or dn_2/dt , respectively, are zero. It is easily seen from equations (3) that when $dn_1/dt = 0$, then $k_1 - n_1 - \alpha n_2 = 0$; therefore $\alpha = (k_1 - n_1)/n_2$. Similarly, $\beta = (k_2 - n_2)/n_1$. The values of k_1 and k_2 reached by *Tribolium* (n_1) and *Oryzaephilus* (n_2) living alone in various media, and of α and β calculated from populations of the two species which were competing in the same media, are shown in table 14 (a).

The values of the same variables for *Tribolium* (n_1) and *Rhizopertha* (n_2) populations competing in wheat are shown in table 14 (b). The values of α and β obtained from tables 1, 2 and 10 were calculated at the final position of equilibrium reached between the two species. Here of course $dn_1/dt = dn_2/dt = 0$. The values of β obtained from tables 6 and 8 and § III (c) were calculated at the points where dn_2/dt first became zero. The value of n_1 and these points represents the number of *Tribolium* necessary to prevent further increase of *Oryzaephilus*. It was impossible to estimate α accurately: all that can be said is that it must have a small positive value. The values of k_1 , k_2 , α and β obtained from each population lead to the inequalities shown in the last column of this table. The outcome of competition in each experiment corresponded to that required by these inequalities (see

TABLE 14. THE VALUES OF k_1 AND k_2 REACHED BY POPULATIONS OF *TRIBOLIUM*, *ORYZAEPHILUS* AND *RHIZOPERTHA* LIVING ALONE IN 10 g. OF VARIOUS RENEWED MEDIA; AND OF α AND β CALCULATED AT POINTS WHERE dn_1/dt OR dn_2/dt , RESPECTIVELY, WERE ZERO, WHEN *TRIBOLIUM* WAS COMPETING WITH EITHER OF THE OTHER TWO SPECIES IN THE SAME MEDIA (SEE EQUATION (3), TEXT AND FIGURE 10)

experiment	k_1	k_2	k_1/k_2	k_2/k_1	$\frac{k_1 - n_1}{n_2} = \alpha$	$\frac{k_2 - n_2}{n_1} = \beta$	equilibrium conditions
(a) <i>Tribolium</i> (n_1) and <i>Oryzaephilus</i> (n_2)							
table 1 (wheat):							
adults	425	445	0.96	1.05	$\frac{425 - 360}{150} = 0.4$	$\frac{445 - 150}{360} = 0.8$	$\alpha < k_1/k_2$, $\beta < k_2/k_1$, both species survive
tables 4-6 (fine flour):							
all instars	320	440	0.73	1.38	0*	$\frac{440 - 51}{107} = 3.6$	$\alpha < k_1/k_2$, $\beta > k_2/k_1$, n_1 exterminates n_2
table 8 (fine flour and 2 mm. tubing):							
all instars	650	500	1.3	0.77	0*	$\frac{500 - 50}{130} = 3.5$	$\alpha < k_1/k_2$, $\beta > k_2/k_1$, n_1 exterminates n_2
tables 9-10 (fine flour and 1 mm. tubing):							
adults	175	400	0.44	2.3	0*	$\frac{400 - 80}{175} = 1.8$	$\alpha < k_1/k_2$, $\beta < k_2/k_1$, both species survive
all instars	420	930	2.2	4.5	0*	$\frac{930 - 140}{420} = 1.9$	
§ III (c) (coarse wholemeal flour):							
adults	420	400	1.05	0.95	0*	$\frac{400 - 23}{133} = 3.3$	$\alpha < k_1/k_2$, $\beta > k_2/k_1$, n_1 exterminates n_2
(b) <i>Tribolium</i> (n_1) and <i>Rhizopertha</i> (n_2)							
table 2 (wheat):							
adults	425	338	0.8	1.5	0*	$\frac{338 - 35}{420} = 0.7$	$\alpha < k_1/k_2$, $\beta < k_2/k_1$, both species survive

*. α must have a small positive value, but this method of calculation is too crude to detect it.

Crombie 1945*b*), and where the initial concentrations varied (tables 1, 2, § III (a)) this had no effect upon the concentrations of each species at which equilibrium was reached (figure 10). Furthermore, the values of α and β obtained from equations (3) for *Tribolium* (n_1) and *Oryzaephilus* (n_2) competing in fine flour were surprisingly close to those given above which were calculated (on the basis of somewhat arbitrary but reasonable assumptions) from direct observation of competition in this medium. The point to point correspondence between equations (3) and the experimental data was usually not very good. Two such calculated curves are shown in figure 10. The method of obtaining such curves is described in a previous paper (Crombie 1945*b*). For that (figure 10*a*) fitted to the data in table 1 (c) the following

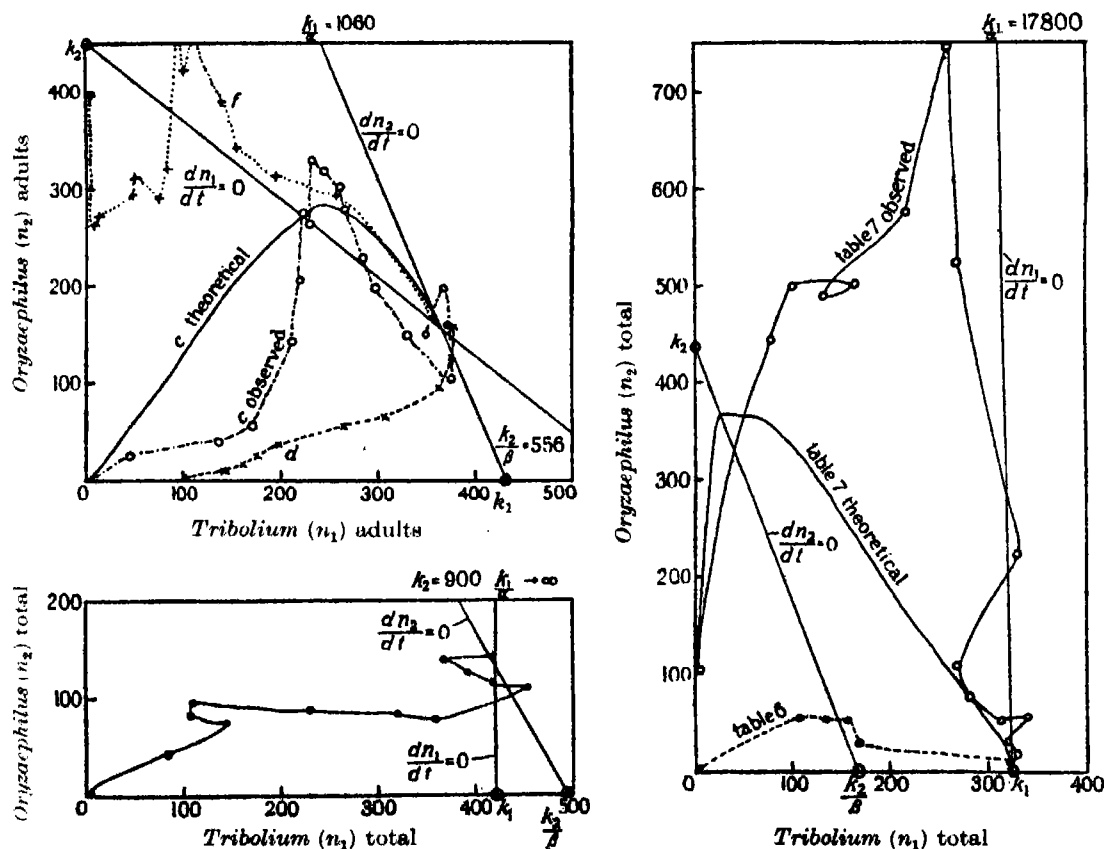


FIGURE 10. Diagrams illustrating the competition between *Tribolium* (n_1) and *Oryzaephilus* (n_2) in various renewed media. (a) *Wheat*. The curves (c, observed), (d) and (f) correspond to the data in table 1 (c, d, f). The curve (c, theoretical) was calculated from equations (3) with constants of the values shown in table 14. (b) *Fine flour with 1 mm. bore glass tubing*. The curve corresponds to the data in table 10 (totals). (c) *Fine flour*. The curves correspond respectively to the data in tables 6 and 7 (totals). The theoretical curve for table 7 was calculated from equations (3) using the values $\alpha = 0.018$ and $\beta = 2.7$, obtained from direct observations of the competition between these two species. The other constants were $k_1 = 425$ and $k_2 = 445$ (table 14).

approximate values were obtained by fitting logistic equations to the data in table 1 (a) and (b) respectively: $b_1 = 0.06$, $b_2 = 0.07$. The values of the other constants are shown in table 14. For the curve fitted to the data in table 7 (shown in figure 10 (c)) the following approximate values were obtained by fitting logistic equations to the total population in tables 4 and 5, respectively: $b_1 = 0.087$, $b_2 = 0.076$. The values of the other constants were: $k_1 = 320$, $k_2 = 440$, $\alpha = 0.018$, $\beta = 2.7$. As the population growth of these species does not strictly obey the 'logistic' law, one could hardly expect equations (3) more than merely to represent accurately the outcome of competition between them.

In Park, Gregg & Lutherman's (1941) study of the competition between the beetles *Tribolium confusum*, *Gnathocerus cornutus* and *Trogoderma versicolor* in a medium of fine flour, coarse rolled oats and yeast, the outcome of competition often depended on the initial concentrations of the competing species. Thus, when *Tribolium* got well established it always drove out *Gnathocerus*, but when the *Tribolium* adult population was reduced to a low value by an epidemic infection it was exterminated by superior numbers of *Gnathocerus*, which apparently eats its eggs, small larvae and pupae. But if the *Tribolium* adult population were not made by the infection to undergo violent oscillations, it seems probable that it would always exterminate *Gnathocerus*. *Tribolium* always exterminated *Trogoderma*, irrespective of initial imaginal densities, at a rate roughly proportional to the initial concentration of *Tribolium*. With equal or superior initial densities of *Gnathocerus* this species always exterminated *Trogoderma*. *Gnathocerus* seems to have inhibited *Trogoderma*'s pupation and emergence (perhaps by predation). When *Trogoderma* had the initially higher density, *Gnathocerus* was exterminated. The values of the constants k_1 , k_2 , α and β calculated from the data in their tables 15 and 16 usually correspond to the fourth condition of equilibrium for the Lotka-Volterra simultaneous equations (Crombie 1945), where the inhibition of each species by the other is greater than its auto-inhibition, and it is initial densities which chiefly determine which species will exterminate the other. But the nature of the competition between these species is not yet fully understood.

VI. DISCUSSION

'It is good to try in imagination to give any one species an advantage over another. Probably in no single instance should we know what to do. This ought to convince us of our ignorance on the mutual relation of all organic beings; a conviction as necessary as it is difficult to acquire. All that we can do, is to keep steadily in mind that each organic being is striving to increase in a geometrical ratio; that each at some period of its life, during some season of the year, during each generation or at intervals, has to struggle for life and to suffer great destruction' (Darwin, *Origin of Species*, p. 78). The comparison of the rates of oviposition with the rates at which adults emerged shows that there was a mortality of over 99 % in the immature stages in saturated populations of three of the four species

of insect studied and of about 90 % in saturated populations of *Sitotroga cerealella* (Crombie 1942, 1943, 1944, 1945). The immature stages were therefore subjected to an immense struggle for existence (cf. Clements & Shelford 1939; Graham 1939). This is reflected in the competition between two different species, which always depended mostly upon the destruction and resistance to destruction of the immature stages of one species by the other. It can be shown (Crombie 1943, p. 95) that, theoretically, if two species with identical needs and habits colonize the same limited environment one will eliminate the other unless density-independent factors keep the population low enough to eliminate competition. Of the insect species studied in these papers, *Rhizopertha* exterminated *Sitotroga* because their larvae had the same needs and habits, and *Tribolium* eliminated *Oryzaephilus* except from environments in which the immature stages of the latter were specially protected. Species whose needs and habits differed sufficiently survived together in the same environment. Thus *Oryzaephilus* was able to survive with either *Rhizopertha* or *Sitotroga*, and *Rhizopertha* with *Tribolium*. The same principle operates among the organisms inhabiting sewage described by Lloyd, Graham & Reynoldson (1940) and Reynoldson (1943), and in the competition between hymenopterous parasitoids described by Parker (1933) and Smith (1933). According to the latter author the larva of the hymenopteran *Scutellista cyanea* preys on the eggs of the black scale *Saissetia oleae*, which are found beneath their parent's body. When the former insect was introduced from South Africa into California the native hymenopteran, *Tomocera californica*, which has almost identical habits with *Scutellista cyanea*, its larvae also feeding on the eggs of *S. oleae* beneath the parent scale (Clausen 1940, p. 210), was almost although not entirely eliminated.

As already pointed out (Crombie 1945), this principle may affect the characteristic fauna and flora of a particular locality in two ways (Carpenter 1939; Clements & Shelford 1939). It may determine the abundance and distribution of individuals of the same biotype in biotic communities. This is well illustrated by the discovery of Elton (1944 *a, b*), based on a study of fifty sample surveys of animal communities in widely different habitats and parts of the world, 'that species belonging to the same genus tend strongly not to occur in the same area of any major habitat at the same time'. He suggests that 'this is attributable to competition for resources available in a particular ecological niche'. Species of the same genus are likely to have the same needs and habits. 'It would follow that the successful introduction of a new species will usually cause the decrease, often disappearance, of one of the species already present.' Possibly the rather constant relationship discovered by Fisher, Corbett & Williams (1943) between the number of species and the number of individuals in a random sample of an animal population may be capable of an analogous explanation (cf. Graham 1933). The second important operation of the above principle may be to lead by 'sympatric speciation' (Mayr 1942) to evolutionary change (Huxley 1942; but cf. Hogben 1940). Sympatric species are those which occur together in one area, as opposed to allopatric species which

replace each other geographically. These problems will perhaps best be solved by the study of natural populations by methods such as those used by Jackson (1936), Gordon (1939) and Dowdeswell, Fisher & Ford (1940).

I wish to thank Dr A. D. Imms, F.R.S. and Dr W. H. Thorpe for some helpful suggestions.

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Reflex conduction in the giant fibres of the earthworm

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The present work confirms the conclusion of Friedländer and others that the giant fibres mediate the end-to-end shortening reaction in the earthworm. The chief concern has been to investigate Stough's claim that the median giant fibre conducts impulses only in the direction from head to tail and the lateral giants only in the reverse direction. Two methods have been employed.

(a) The nerve cord was exposed at each end of the worm, and electrical records taken simultaneously from the two extremities when the surface of the worm was touched at different places. The results were usually a train of impulses in one or other giant fibre, and it was found that whenever an impulse appeared at one end of a given fibre, it always appeared at the other end of the same fibre. Each fibre, therefore, when it conducted at all, always conducted in both directions. Sensory nerves from the head appeared only connected to the median giant, since stimulation anterior to the clitellum never resulted in lateral fibre activity. Similarly, the tail appeared only to join with the lateral giant fibres.

(b) Stough's own method was used, and his observations confirmed, extended and re-interpreted. Either the median or both lateral fibres were divided in one segment. The success of this operation could be judged by leading off the giant fibre responses from the undissected worm (figure 5). Next day, when the worm had recovered, the shortening reflex was observed when the worm was touched at the head, the tail, or in the middle. The shortening was either throughout, or was arrested at the operation site, depending upon whether the active giant fibre was the intact or the damaged one. The results are summarized on p. 119. From both the head and the tail Stough's observations are confirmed, and it is agreed that impulses from the head are conducted back by the median giant alone. The absence of impulses in the laterals might be due to contrary one-way conduction as Stough assumes, or to the absence of their sensory connexion with the head. But (a) above shows that the latter is correct, and the same must be concluded from touching the middle region of the worm, which apparently Stough did not do, for this part connects with the lateral giants, and thus affords a demonstration that these fibres may also conduct antero-posteriorly.

The difference in function of the separate giant fibres, therefore, is probably related to their difference in sensory distribution.

From the time of Friedländer (1894), it has been generally accepted that the function of the giant fibres of the earthworm is to mediate the end-to-end reaction which is often seen when a worm is touched. Since, apparently, only one kind of shortening has been described, it is not obvious why there are three parallel giant

fibres instead of simply one, and the object of this paper is to see whether any light can be thrown on this matter by electrical records taken from the giant fibres during reflex activity. In particular, we shall examine the claims of Stough (1926, 1930) that the median giant can only transmit impulses towards the tail and the lateral giants only towards the head, as this appears to be the only hypothesis which differentiates between the functions of the three fibres. First, however, it was necessary to establish the reliability and significance of the electrical record obtained from the whole nerve cord (Rushton 1945).

If the isolated nerve cord be excited electrically, two potential waves of about 5 mV are recorded. These show an all-or-nothing relation to the stimulus and are conducted at about 10 and 20 m./sec. respectively. Various tests show that they are due to two independent excitable structures, the more slowly conducted wave being associated with a longer chronaxie. Micromanipulation allows the faster wave only to be abolished by damage applied to the median giant, and the slower wave only by damage to both laterals in the same segment. Further proof that the giant fibres conduct these waves is afforded by transection of the whole nerve cord except for the giant fibres, for this still allows both the waves to pass. Section of the lateral giants alternately at intervals of ten segments apart still allows the slower wave to be propagated at full size, and this and all other observations are independent of the direction of propagation.

These results, which confirm and amplify those of Eccles, Granit & Young (1933), strongly point to the conclusion that the fast wave is the action potential of the larger median fibre and the slow wave that of the two lateral fibres which must interconnect through a conducting bridge at least once every ten segments. Moreover, all these three fibres may conduct as effectively in one direction as the other.

This is perhaps surprising in relation to the fact established histologically by Stough (1926) and confirmed by Smallwood & Holmes (1927) that the giant fibres are not single neurones. In every segment each giant fibre is in continuity with a cell body, and there is a transverse membrane interrupting its continuity with the fibre in the next segment. This harmonizes with the experiments of Bovard (1918) who cut the cord and found no degeneration of the giant fibres.

When two nerve fibres make functional union the junction is usually called a synapse, and this is the name given by Stough to his transverse membranes. It should be noted then that these synapses are unusual in that they conduct invariably and in both directions, and they have so little synaptic delay that 1 msec. may suffice to pass some forty of them in succession and to traverse 2 cm. of fibre as well.

Though the work just quoted substantiates many of the claims of Stough, it runs quite counter to his belief that the fibres can only conduct in one direction, a conclusion which was hardly deducible from his staining reaction, itself denied by Smallwood & Holmes. In quite a different category, however, is Stough's claim (1930), derived from experiment, that in reflex activity impulses always run antero-posteriorly in the median giant, and in the opposite direction in the laterals. This result would appear to be analogous to the familiar condition in vertebrate reflexes,

where both sensory and motor nerves conduct in a single direction though physically capable of conducting in both. It was therefore scarcely admissible to use this observation to support the claim that the giants *can* only conduct in one direction.

But on closer consideration there is an important difference between the vertebrate reflex and what is claimed of the giant fibre. For in the vertebrate arc in general the neurones are joined end to end, but in the worm the giant fibre response is obtainable by touching the body anywhere, and thus the sensory segmental nerves probably join a giant at various places along its course. If now, say, in the middle of the worm an impulse from a sensory nerve can pass to a lateral giant, and if, as is claimed, the junction is of a kind which will allow the impulse to turn towards the head and not towards the tail, then this junction will prove a very interesting phenomenon.

The first object then will be to study the effect upon the giant fibres of the reflex arising from touching various points upon the worm's surface, and in particular to note which giant fibre is stimulated and in which direction or directions the impulse is conducted.

THE DIRECTION OF REFLEX CONDUCTION IN THE GIANT FIBRES

The aim of this experiment is to analyse the reflex activity of the giant fibres, by obtaining simultaneous records from both extremities of the ventral nerve cord. In general, the wave in the median giant will differ in appearance from that in the lateral and may be identified. It is easy then in every record to tell which fibre conducts the impulse from the stimulus site to the head, and which to the tail. There are a good many technical difficulties in getting satisfactory results, but these may usually be overcome as follows.

Dissection. The worm is anaesthetized by dropping into 10 % methylated spirit in water for 5–10 min. If the anaesthesia is too light, dissection is difficult and autotomy very liable to occur. If too deep, the giant fibre reflexes are hard to elicit in the subsequent experiment. After washing off the excess spirit from the skin, some 15 mm. of the posterior end of the cord is isolated, ligated and cut posteriorly, and then tucked back into the body cavity for protection whilst the anterior end is prepared. A mid-ventral incision is made just through the body wall from the 14th segment to the first, and the worm is pinned down on its back with nearly all its body floating on mercury, where it cannot get a grip to aid autotomy. The cord is ligated and cut just below the suboesophageal ganglion, and then very carefully isolated up to the point where it enters the mass of the seminal vesicles. It is important not to cut through one of the hearts which are apt to protrude suddenly through the incision. In favourable cases this dissection took 15 min.: the worm was then transferred to the recording turn-table.

Recording turn-table. It is clearly necessary to fix each end of the worm to a pair of recording electrodes, but apart from this it is desirable to allow the animal as

much freedom as possible. If it is pinned down and then stimulated to give reflex responses, it usually autotomizes, often in many places. A turn-table round which the worm could crawl (on the tread-mill principle) proved satisfactory.

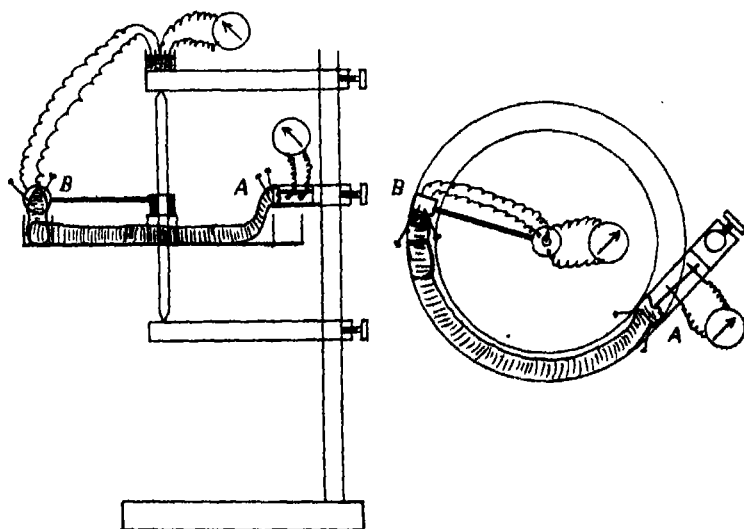


FIGURE 1. Recording turn-table. Description in text.

The idea is indicated in figure 1. The turn-table is light and mounted on conical bearings which will turn very easily. The worm lies in the circular slot and occupies about half the circumference. Its posterior end rises from the slot to be attached, dorsal aspect uppermost, to a fixed platform *A*. The anterior end is attached to the cork *B* mounted on a radial arm freely movable about the axis independent of the turn-table. Suppose for a moment that there was no posterior fixation and that the tail just lay in the slot. Then the worm can obviously crawl round and round the turn-table pushing the arm *B* before it, and being thereby guided to keep in the slot. The only difference which results from the actual arrangement is that, owing to posterior fixation, the worm drags the turn-table round backwards instead of itself going round the turn-table forwards. This will hardly affect the freedom of the worm, but greatly simplifies the problem of electrical recording.

This is made by a fixed pair of electrodes mounted on *A*, and a second pair mounted on *B* making contact with the fixed circuit through concentric mercury cylinders coaxial with the turn-table. In this way good contact is made with each end of the nerve cord despite fairly free movement of the worm. In some experiments the head was fixed to *B* and the tail to *A*.

Electrical recording. Each pair of electrodes could be connected to one of a pair of differential amplifiers (Matthews-Tönnies type), each deflecting one spot of a double-beam cathode-ray tube. Thus when both pairs of electrodes were so connected, simultaneous records could be taken from head and tail. Each pair of

electrodes could alternatively be connected to a stimulating circuit giving a brief condenser shock synchronized with the time-base.

A recording speed of 30 cm./sec. was used to enable the wave form to be distinguished. This was done economically by photographing single sweeps of the time-base operating synchronously on the two beams. Since, however, the time of traverse is only $\frac{1}{15}$ sec., it is clearly necessary to adjust the sweep nicely to synchronize with the reflex response. This was achieved by triggering the sweep from a thyatron whose grid was normally biased so as just to withhold the discharge. Thus when it was earthed even through 5 megohms, the thyatron discharged and a linear traverse flashed across the screen. Now the worm lies in an earthed metal slot, and its reflexes are to be elicited by touching the body surface with some object. Our problem is therefore solved by using as object the free end of this 5-megohm resistance, for it elicits the reflex by contact, and simultaneously earths the grid and triggers the sweep.

Despite the 5-megohm resistance there will be a small current flowing from the worm to the wire, but this is likely to be a negligible contribution to the stimulus for the following reasons:

(a) Though the current must be independent of the firmness of touching, discharges from the giant fibre were absent in very light touches and they increased with the force of contact.

(b) If the stimulating object was a non-conductor, e.g. a glass rod, the discharges were similar to those recorded. They could not be clearly photographed, but they could be appreciated by clicks in a loud speaker and by direct observation of the continuously running time-base with automatic fly-back.

(c) On touching my own (earthed) tongue or lips with the 5-megohm wire, I could not detect any trace of shock, though I naturally experienced the sense of touch.

Results. Figure 2 shows a typical series of results. Each frame shows two faint lines which are due to the fly-back of the sweep, and are to be disregarded, and two heavy lines, the upper of which is the record from the tail and the lower from the head.

Frame *a* is the magnetic deflexion due to 500 cyc./sec. and shows the speed and degree of linearity of the time-base. In *b* the head electrodes were switched from amplifier to stimulator, and the record from the tail shows the effect of a single shock. The result is precisely the same as in all the experiments with the isolated nerve cord (Rushton 1945) and may be similarly interpreted. The faster wave which travels in the median giant fibre is here almost monophasic, whereas the slower wave in the lateral giants is clearly diphasic. Frame *c* shows the record from the head when the shock is applied to the tail. Both waves are nearly monophasic, but the median is easily recognized as being about three times the amplitude of the other.

Frames *d*, *e* and *f* are the responses from tactile stimuli applied approximately to segments 20, 25 and 30 respectively. It is clear that the responses are repetitive, that they appear at both extremities in the median fibre, and that the lateral giants are not involved. Frame *g* shows no response in any giant fibre. As is well known, the middle region of the worm is the least sensitive, and no waves were

found in the present series between the clitellum and about 3 cm. from the posterior site of operation, i.e. 5 cm. from the tail. Frames *h*, *j* and *k* show the responses arising from 5, 4 and 3 cm. from the tail respectively. In these cases the repetitive responses appear at both extremities of the lateral giants and the median is not involved. Frames *l* and *m* are the final repetition of *a* and *b*, to verify that the wave form has not changed.

Now if Stough is right in supposing that in these reflexes each giant fibre conducts in one direction only, there is only one way to explain the invariable appearance of the wave at both extremities of the same fibre. It is that the impulse is conveyed by other nerves from the site of the stimulus to the anterior end of the median, or the posterior end of the laterals, and is then conducted down the whole length of the giant fibre. There are several reasons against this explanation, and it will suffice to note that the recorded reflex waves are similar to those in frames *b* and *c* and are not inverted. This must mean that every wave in figure 2 is propagated across the recording electrodes in the direction away from the centre of the worm.

If then one rejects, as clearly one must, the idea of one-way reflex conduction in the giant fibres, there no longer appears anything striking about the junction of the sensory segmental nerve with the giant fibre. Impulses pass from the former and are conducted in both directions along the giant, and arrive at the two ends with a latency difference depending upon the difference in conduction distance. In records *d*, *e* and *f* the worm was touched anterior to the clitellum and in each case the impulse reached the head before the tail. The reverse is the case in *h*, *j* and *k*, where the stimulus was close to the posterior end. The latency difference is more obvious with the lateral giants because they conduct much more slowly as is seen from the shock-spike intervals in frame *b*, which give the time for conduction over the whole length of each fibre. There is thus a reasonably good correspondence between the latency difference observed and that calculated upon the assumption that the impulse arises in the giant fibre at the level of the stimulus and is conducted in both directions at the constant velocity shown in *b*.

This, however, applies only to the first wave of the series. In *d*, for example, though the first wave arrives earlier at the anterior end, the sixth wave appears earlier at the tail. It is known that when one impulse follows closely upon another its conduction speed is diminished, and Matthews has drawn attention to this in his records of sudden discharges in the nerve from a muscle spindle (1931). In the present case this slowing of conduction seems more pronounced at the anterior extremity of the median giant fibre than elsewhere.

It has been concluded from figure 2 that whenever an impulse appears at one end of a giant fibre it also appears at the other. This was found without exception in 500 pairs of waves which satisfied the condition that the final records (*l*, *m*, figure 2) reproduced the initial ones (*a*, *b*). When this failed the series was rejected. But further work has revealed one worm which showed three exceptions. As these run contrary to the conclusions of the present paper, they are given in their setting in figure 3.

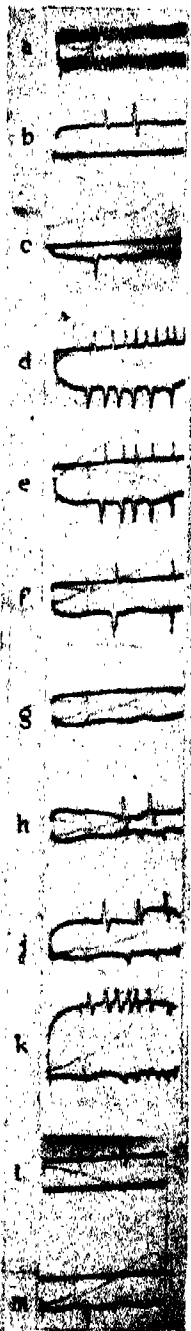


FIGURE 2

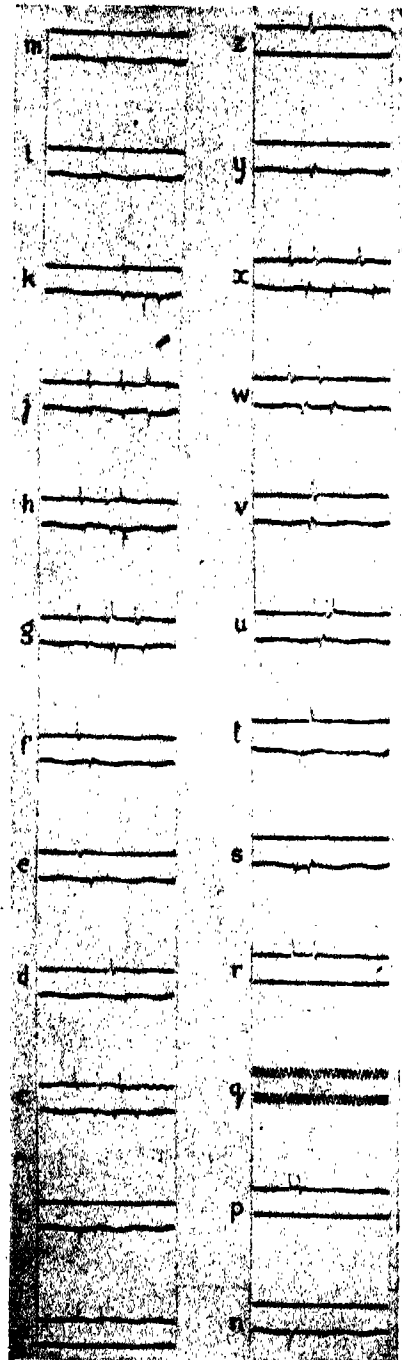


FIGURE 3

FIGURES 2, 3. Action potentials from giant fibres. Upper line in each frame is the record from the posterior end of the cord; lower line is the record from the anterior end. Details of figure 2 are described on p. 113. Details of figure 3 are described on p. 116.

The experiment was essentially the same as that of figure 2 except that this time the head of the worm instead of the tail was fixed to platform *A*, figure 1. Frame *a* is a record from the tail when a shock is applied to the head; *b* is the corresponding record from the head. In frames *c* to *m* the worm was prodded with the wire at points starting near the tail, and each in succession some 8 mm. nearer the head, *m* being just anterior to the clitellum. Points nearer the head gave no response and are omitted. Frames *p* and *n* repeat *a*, *b* and *q* is the 500 cycle time. The nerves were moistened and readjusted on the electrodes with the slight changes in wave form seen in *r* and *s*, and *t* to *x* gives a further series due to prods starting this time at the head and proceeding caudally (some records are omitted). Frames *y* and *z* repeat *r* and *s*.



FIGURE 4

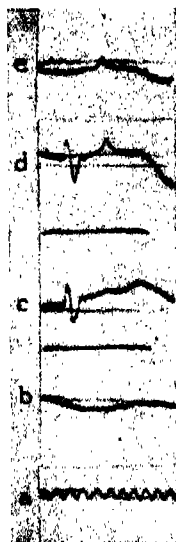


FIGURE 5

FIGURE 4. Diagram of a worm with the median giant fibre divided at the arrow. Sensory nerves in the shaded region are connected chiefly to the lateral giant fibre system.

FIGURE 5. Records taken from the surface of an anaesthetized undissected worm. (*a*) 1000 cycles time scale, (*b*) just subthreshold shock, (*c*) shock just threshold for the median fibre response, (*d*) shock increased to stimulate lateral fibres also, (*e*) successful puncture of the median abolishing the median wave, but allowing the lateral to persist.

This record differs from figure 2 in that the middle region is not inexcitable, and frames *g*, *h* and *j* show that both median and lateral giants may be simultaneously active, and conduct independently in both directions. Record *k*, however, taken from about the posterior border of the clitellum, shows two median waves appearing at the head which do not arrive at the tail. Frames *n* and *p*, nevertheless, prove that conduction is possible throughout both fibres in both directions. Record *u* again shows one-way conduction of the median, but this time the impulse arrives

at the tail only. It is natural to suspect a critical state of partial conduction in the region of this stimulus site. If *k* were anterior and *u* posterior to this place it might account for the two directions of one-way conduction. But, judging by the latency difference of the lateral fibre waves, *k* is nearer the tail and *u* nearer the head, so I can see no obvious explanation. Unfortunately, I was not aware of the anomaly until examining the record some hours after the experiment was finished.

This much is clear. First, the median fibre was not in a normal state of conduction, because by the time records *y* and *z* were obtained it had lost through-transmission altogether, and it never regained it thereafter. Secondly, the phenomenon, whatever it may be, is exceedingly rare in the conditions of my experiments, for I have never seen it before or since. Thirdly, it lends no support to Stough's concept of one-way conduction, for the very anomaly has presented itself once as conduction forwards and once backwards. In the absence of further evidence, therefore, it seems best to treat this as some unexplained imperfection of conduction, and ignore it.

REFLEX CONTRACTION WITH A DIVIDED GIANT FIBRE

The foregoing observations place us in a better position to understand the conditions of Stough's experiment (1930). He cut through (say) the median giant fibre and, after the worm had recovered from the operation, he found that the shortening reaction elicited by touching the tail was normal, but when the head was touched, the shortening extended only as far as the operation site. His immediate conclusion, which I confirm, is that the 'head reflex' is conducted by the median giant fibre (up to the damaged point), and the 'tail reflex' is conducted by the laterals (throughout).

There are two possible explanations of this. Either the sensory nerves of the head are in physiological connexion with the median and not the lateral giants, or they are connected to both, but impulses can only pass backwards in the median and forward in the laterals. Stough accepts the latter interpretation without, apparently, considering the other, and does not mention touching the worm in the middle, which allows us to settle the matter. But from figures 2 and 3 it appears that the head is only connected to the median giant, and that impulses can pass in both directions, hence the first interpretation above is clearly true, and the second is certainly false.

It can now be seen how Stough's own experiment could have been conducted so as to distinguish between the two interpretations. For if the median giant (say) is divided half-way between the clitellum and the tail (figure 4), then in the shaded area between the clitellum and the cut, according to figure 3, stimuli should affect chiefly the lateral giants and hence elicit a shortening reaction throughout the full length of the worm just as if the tail were touched. According to Stough, on the other hand, the backwards conducted impulses must necessarily flow in the median,

and hence the shortening must be confined to the region anterior to the site of operation, just as if the head were touched. The experiment now to be described, therefore, is to elicit the shortening reflex from the head, the tail, and the middle region of the worm after dividing the median or lateral giant fibres. In anticipation it may be stated that in the reflexes from the head and the tail the observations of Stough are confirmed, but the reflex from the middle region refutes his interpretation.

Operation. Stough took great care to see that his giant fibre was completely divided and that no other structure was damaged. I was less exact and employed one of two methods.

(a) After the usual alcohol anaesthesia an incision was made about 1 cm. long in the line of the ventral setae in the region shown in figure 4. The nerve cord was exposed and isolated for several mm. with care to avoid damage to the ventral blood vessel. The tiny electric bulb of an ophthalmoscope was placed below the nerve cord which was viewed, dorsal side uppermost, at binocular magnification of 15 diameters. This transillumination allowed excellent observation of the giant fibres, and the median or both laterals were damaged by pricking and tearing the upper surface with a fine needle. The incision was then closed by suture, and the worm left in the dark in a clean closed jar containing wet cotton-wool. Experiments were made during the next few days.

(b) After anaesthesia the worm was floated on mercury (electrically earthed), with the head and tail raised above the surface. A strong electric shock applied to the tail stimulated the giant fibres, and in the absence of much muscular response (due to deep anaesthesia) the action potentials of the giant fibres may be led off from the surface of the worm by electrodes applied to the anterior end (Rushton & Barlow 1943). With the shock synchronized with the time base each action potential appears in a fixed place on the tube, and may be identified by shape and position. Now a transverse incision, 3 mm. long, is made in the appropriate place on the ventral surface of the worm, to expose the nerve cord for 1 mm., but the giant fibres cannot be seen since they run on the dorsal aspect of the cord. A fine needle is plunged through the centre of the cord and out beyond, and the worm replaced on the electrodes. Sometimes both waves were still present, whereupon another puncture was made. Sometimes both waves were abolished, and the worm was rejected. In favourable cases the median wave was abolished while the lateral persisted. No suture was applied, and the worm kept as in (a).

An example is shown in figure 5. Frame *a* is 1000 cycles time scale, *b* shows the effect of a just subthreshold shock, in *c* it is just threshold for the median wave, and in *d* for the laterals. Frame *e* shows the effect of a successful median puncture.

Observation. The best way of testing the reflexes was to leave the worm in a moist flat dish in the dark for an hour. The motor response to touching is thereby greatly enhanced as it is when worms are found in the open in the night time. Then, after dark-adapting oneself, the contraction is observed by the light of a photographic safe-lamp. The worm is touched once and then allowed a further period of

a quarter of an hour to rest before touching again. In these circumstances the shortening reaction is usually very definite, and may be described as follows.

Median divided. (i) Touching the tail gives the normal shortening of the whole worm with the exception of segments denervated at the operation site in method (a). In particular, the segments just posterior to the operation site become more crowded.

(ii) Touching the head gives shortening of the worm down to the operation site, posterior to which the body remains flaccid and is passively extended by the anterior pull, so that, in contradistinction to (i), these segments become less crowded.

(iii) Touching the middle of the worm is apt to give no response at all, consistent with the relative insensitivity of this region. When there is a brisk response, however, it is like (i), and there is no increased spacing of the posterior segments. The observation is thus inconsistent with Stough's interpretation.

Laterals divided. (i) Touching the head gives the normal shortening of the whole worm except for denervated segments.

(ii) Touching the tail gives a shortening only posterior to the operation site.

(iii) Touching the middle of the worm gives shortening only anterior to the operation site.

Here again touching in the middle discriminates against the explanation of Stough, for reflexes in both (ii) and (iii) are transmitted only as far as the operation site and hence are carried by the divided laterals but in opposite directions, while reflexes (i) and (iii) though both transmitted antero-posteriorly are affected differently at the point of nerve division, showing conduction in (i) to be median and (iii) lateral.

In some experiments I 'listened in' to the giant fibre responses accompanying these reflexes. A slab of paraffin wax had a circular groove cut in it 5 cm. in radius and 8 mm. wide and deep. Upon the floor of this groove were embedded twelve equally spaced radial copper wires with the upper surface scraped bare, each of which passed outwards to enter a mercury cup open to the surface. So any pair of radial wires could be connected to an external circuit without disturbance, by placing the external leads into the appropriate mercury cups. The groove was moistened, and the worm introduced and imprisoned by placing a glass cover on top. After leaving in the dark a suitable time, it was viewed by dim light. If the worm was extended at rest the cover was removed, and two pairs of leads connected through the mercury, one pair to a region of the worm anterior and one pair posterior to the operation site. Each pair connected to a separate amplifier and the output went one to each ear-piece of a pair of head telephones.

The reflexes were elicited in the ordinary way by touching some part of the worm (with an electric insulator) and the nature of the reflex contraction noted by eye. At the same time the two earphones gave information of the giant fibre responses by a series of distinct clicks amidst a good deal of other noise. If the particular fibre involved was the damaged one, the clicks were heard in one ear only. If it

was the intact one, they were heard in both ears. It thus was possible simultaneously to perceive both the identity of the fibre, the nature of its discharge, and the type of motor response resulting.

As these results were consistent with the interpretations which have been set forth earlier in this paper, they need no further comment.

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Reversible adsorption of proteins at the oil/water interface

I. Preferential adsorption of proteins at charged oil/water interfaces

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The behaviour of positively and negatively charged oil-in-water emulsions, stabilized with hexadecyl trimethyl ammonium bromide and sodium hexadecyl sulphate respectively in the presence of protein solutions has been studied.

Under certain conditions proteins will adsorb to a charged oil/water interface. When finely dispersed oil-in-water emulsion was used to provide this oil/water interface, adsorption of protein resulted in flocculation of the oil droplets.

Flocculation of emulsion on the addition of protein is pH conditioned and occurred on the acid side of the isoelectric point of the protein with negatively charged and on the alkaline side with positively charged oil globules. No flocculation occurred on the alkaline side of the isoelectric point with a negative emulsion or the acid side with a positive emulsion.

The amount of protein required to cause maximum clarification of the subnatant fluid corresponded with that needed to give a firmly gelled protein monolayer at the interface, namely, 2.5 mg. of protein/sq.m. of interfacial area. With that amount of protein the flocculated oil globules remained discrete and no coalescence or liberation of free oil occurred. If only 1 mg. of protein/sq.m. of interfacial area was added, flocculation was followed by rapid coalescence of oil globules and liberation of free oil. If smaller amounts still were used, no visible change in the dispersion of the oil droplets could be seen macroscopically. With greater amounts than 2.5 mg./sq.m. of interfacial area, up to ten times the monolayer concentration was adsorbed to the interface.

Sodium chloride affected the flocculation range, and instead of the clear-out change-over between the positive and negative interfaces at the isoelectric point of the protein, overlapping occurred. 5 % sodium chloride shifted the flocculation point about 1 unit of pH. The addition of sodium chloride also altered the point of maximum clarification. Thus with haemoglobin the maximum clarification point was shifted from 2.5 to 1.7 mg./sq.m. of interfacial area by the addition of 1 % sodium chloride.

The adsorption of protein on to charged oil/water interfaces was reversible. This was best demonstrated with haemoglobin. Thus, haemoglobin was adsorbed at pH 5.0 to a negative emulsion—the red floccules were washed and transferred to a buffer at pH 10. The haemoglobin was released and the emulsion was redispersed.

The effect of adsorption and desorption on the structure of the protein molecule has been studied with haemoglobin. By solubility and colour tests it was shown that the haemoglobin molecule was changed to parahaematin by adsorption and subsequent desorption from a charged oil/water interface.

Molecular weight and shape determinations were carried out on the desorbed protein.

Two proteins have been separated by this adsorption mechanism. This was demonstrated on a mixture of albumin and haemoglobin.

Some applications of the flocculation technique are indicated and the significance of the phenomena described are discussed.

* Sir Halley Stewart Research Fellows.

(This paper has been printed in full in *Proceedings A*, 184, 102.)

Address of the President
Sir Henry Dale, O.M., G.B.E., at the
Anniversary Meeting, 30 November 1945

The annual number of *Obituary Notices of Fellows of the Royal Society* published today, and the names which have just been read to us, remind us of the losses from its Fellowship which the Society has suffered during the year now closing.

I propose on this occasion to proceed next to the presentation of the Medals for 1945.

Awards of Medals, 1945

The COPLEY MEDAL is awarded to Dr OSWALD THEODORE AVERY for his contributions to knowledge of the chemical basis of the specific properties of bacteria, particularly of the types of the pneumococcus. His researches in this field have appeared in unhurried and orderly sequence over the course of a long and distinguished career, and they have furnished a large and essential constituent of the framework now available for a fundamental science of immunochemistry.

We allow ourselves here to claim Avery as Canadian by birth, though with acknowledgment that his life's work has been accomplished in the United States of America, and in the Rockefeller Institute of New York in particular, of which he has held the Membership since 1913.

It was in 1917 that Dochez and Avery demonstrated that cultures of different strains of the pneumococcus yielded different 'soluble specific substances'. From 1923 onwards appeared a remarkable series of papers by Avery, with Heidelberger and other collaborators, in which it was shown that these specific substances had the nature of complex polysaccharides of highly individual characters. These were present in the regular capsular envelope characteristic of the pneumococcus in its virulent forms; and each type of such virulent pneumococci, distinguishable by its immunological specificity, was shown to have its own distinct polysaccharide. Each of these reacted, with a like specificity, with the corresponding immune body. Not that these polysaccharides, the soluble specific substances isolated in chemical purity, had antigenic properties by themselves. It was only when they were artificially linked to proteins foreign to the reacting animal body, or retained their natural linkage with proteins of the bacterial strains producing them, that they elicited, on injection, the appearance in the blood of specific immune substances, causing agglutination or lysis of the corresponding organisms; but, with the immune substances thus evoked, the pure, separated polysaccharides now exhibited the same specific affinities, each forming a precipitate with the corresponding anti-serum.

Here, then, in chemically definite form, were separable, prosthetic, combining groups such as Paul Ehrlich had long earlier envisaged and prophetically named 'haptenes'. Here also was one of the principal foundation stones of a great building of immunological chemistry, which, in the hands of Avery's contemporaries and followers, notably in those of a distinguished fellow-Member of the Rockefeller Institute, the late Karl Landsteiner, has rapidly included an ever-widening range of studies of artificial and natural antigens. Fellows of this Society may recall that last year we were privileged to hear Bakerian and Croonian Lectures, by Professor Haworth and Dr Harington respectively, both dealing, from somewhat different angles, with experiments in immunochemistry, and each contributing its own important extension to a structure of knowledge founded so largely on Avery's pioneer discoveries.

Meanwhile, in the hands of Avery and his co-workers, knowledge of the specific characters of the pneumococci, and of the manner in which these are acquired, had been moving quietly to a new pinnacle of achievement. They had long ago shown that pneumococci, which, in artificial culture, have lost the capsules endowing them with virulence and containing the specific polysaccharides, have reverted to avirulent non-specific types, growing in the rough, wrinkled colonies characteristic of such defective strains. It had been shown also, by the late Fred Griffith, that such a degenerate, non-specific pneumococcus, from whatever specific type it had its provenance, could be induced by cultivation in a medium prepared from a complete, virulent type to reacquire a capsule conferring the corresponding specificity. And now, only last year, Avery, with Macleod and McCarty, has been able to isolate and to characterize a chemical principle acting in minute dosage as the specific stimulus to such a transformation. An unencapsulated, avirulent, typeless pneumococcus derived from a specific strain of type II, responds to this stimulus by acquiring and retaining the capsule and specific polysaccharide, with the virulence and the cultural characters, of a fully specific strain of type III. Here surely is a change to which, if we were dealing with higher organisms, we should accord the status of a genetic variation; and the substance inducing it—the gene in solution, one is tempted to call it—appears to be a nucleic acid of the desoxyribose type. Whatever it be, it is something which should be capable of complete description in terms of structural chemistry.

It has been a matter for rejoicing to his many admirers, friends and followers in many countries that Avery, a veteran now among investigators, should thus, on the eve of his retirement, have attained this new peak of discovery—a fitting climax to a devoted career of such wide influence on the progress of science. Many, we feel with assurance, in his own country and far beyond it, will welcome and approve our award to Dr Avery in this year of the Royal Society's highest recognition, its Copley Medal.

A ROYAL MEDAL is awarded to Professor JOHN DESMOND BERNAL, in recognition of his distinguished contributions to the theory and the applications of X-ray crystal analysis.

Bernal's first important work in this field, published in 1926, was a fundamental study of the basis of the interpretation of X-ray rotation photographs of single crystals; and the methods which he then evolved are still in use. He was largely concerned with the initiative which led to the construction of the International Crystalline Tables, and himself accepted an important share of the editorial work required. Later he became a leader in the application of X-ray crystallography to the elucidation of the structure of highly complex organic molecules. Organic chemists had encountered difficulties, long insuperable, in formulating a satisfactory structure for the sterols. Here Bernal's crystallographic data, indicating the general shape and dimensional limits of the sterol molecule, supplied a key which opened the way to a convincing reconstruction of the polycyclic framework of cholesterol, ergosterol and calciferol in the first place, and eventually to the structural formulation of a vitally important series of gonadal and adrenal hormones, as these, in due course, were isolated and identified. With admirable enterprise he, with his pupils and associates, proceeded to apply the methods of X-ray crystallography to crystals of some of the simpler proteins, as these became available, such as crystalline pepsin and, later, insulin. Then the discovery, by Stanley, that a plant virus, that causing the 'mosaic' disease of tobacco, could be obtained in crystalline form, opened the way for Bernal to apply his technique to elucidate the structure of a protein endowed with such paradoxical characters. He was able to describe the virus units as long, rod-like structures, 1500 Angstroms in length by 150 in diameter, and with an inner regularity in structure fitting a hexagon lattice and, apparently, consisting of cubical sub-units measuring about 11 Angstroms. The readiness with which the long virus units, in a solution, set themselves parallel in a two-dimensional lattice, provided an explanation for the double refraction and other physical properties which such solutions of the virus exhibit.

Just before the war Bernal had published a preliminary note on the structures of haemoglobin and of chymotrypsin. Then, like others, he found his activities diverted to the scientific service of the special needs of the nation and its allies at war. The time is not yet for detailed mention of the important special researches he has carried out during the years immediately past, for the Ministry of Civil Defence, the Combined Operations Command, and other Service Departments, which have successively made claims on his special knowledge and ability. We are glad to know that some of his pupils have been able to keep the thread of his more normal scientific activities unbroken over this interval. Biochemistry, as well as the Physics of his primary discipline, will expect much now from his resumption of personal participation and leadership in a field which he has made so much his own.

A ROYAL MEDAL is awarded to Dr EDWARD JAMES SALISBURY in recognition of his distinguished contributions to plant ecology.

Salisbury's work has had a most important influence in broadening the basis of the study of British plant communities, and in diverting ecological work in this country from an essentially floristic outlook to one in which the habitat and the autecology of individual species have been put in the forefront of interest.

Combining his expert ecological knowledge with a wide acquaintance with cultivated plants and their conditions of growth, Salisbury has shown an exceptional capacity for relating horticultural practices to known physiological and ecological facts, nowhere more strikingly shown than in his *Living Garden*. A similar faculty served the country well in the earlier part of the war in connexion with his manifold activities on the Agricultural Research Council.

Making his first contacts with ecology by a detailed study of the oak-hornbeam woods of Hertfordshire, to which he brought some of the experience gained by co-operation with F. W. Oliver in the investigation of the maritime communities at Blakeney Point, Norfolk, Salisbury soon exhibited a more generalized approach to ecological problems. Examples are furnished by his papers on the calcicolous habit (1920) and on leaching (1922) which traverse a wide field and open up new points of view. Although somewhat different in scope, the investigation on stomatal frequency, supported by a very large mass of data and published in the *Philosophical Transactions* in 1927, is essentially directed to clarifying the ecological picture.

In his presidential address to the Ecological Society in 1929 on the biological equipment of species in relation to competition, a topic which is repeatedly touched upon in his earlier work, attention was drawn to the almost complete lack of information on the reproductive efficiency of the different species of flowering plants. This aspect of a species' equipment had already interested Salisbury for some years, and a number of significant data were given in the course of his address. Despite preoccupation with many other matters, he continued actively to accumulate information on reproductive capacity during the subsequent years, collecting data on the seed production of over 240 British species, which involved the examination of several hundreds of thousands of individual plants. The numerous important conclusions derived from this comprehensive study, which disposes of several fallacies, were published in 1942 in a book, *The Reproductive Capacity of Plants*, which constitutes a landmark in the progress of plant ecology.

In addition to his ecological work, Salisbury is known to his botanical colleagues for earlier work on fossil seeds and for important contributions to the distribution of British plants and to the interpretation of floral morphology. When a man with his knowledge and his love of plants and of the various conditions under which they live and thrive, becomes the Director of Kew Gardens, not only his fellow-botanists but a wider constituency of plant lovers may well find reason for rejoicing. Here we may call to mind the names of two of his distinguished predecessors at Kew, Sir Joseph Dalton Hooker and Sir David Prain, who served this Society also

as President and Treasurer respectively. More recently Sir Albert Seward was Foreign Secretary, but I find no record of a botanist as one of the Society's two Secretaries since Nehemiah Grew was appointed in 1679. In the long, slow rhythm of our activities it might well be time for us now to choose another, and the Council have every confidence that the Fellows will accept their nomination of Dr Salisbury, to whom it is also a special pleasure to hand this Medal, in recognition of his great achievements in botanical research.

The DAVY MEDAL is awarded to Professor ROGER ADAMS of Chicago, in recognition of his distinguished researches in organic chemistry.

Roger Adams, Professor of Chemistry in the University of Illinois, is indoubtedly the outstanding organic chemist in the United States at the present time, and his school, the largest and most vigorous of its kind in that country, is of international repute.

His researches, distinguished by great originality and dogged perseverance alike, embrace a remarkably wide field. In a series of brilliant papers he has described the complete elucidation of the structure of gossypol, a highly complex pigment present in cottonseed. This achievement is all the more noteworthy since several other investigators had failed to make much headway in examining this highly sensitive compound. His experimental skill and theoretical insight have, in recent years, led to notable advances in alkaloid chemistry and in this connexion special mention may be made of his work on the structure of monocrotaline, the toxic constituent of various *Crotalaria*. Adams has been responsible for pioneering research in attempts to find synthetic substances antagonistic to the leprosy bacillus and other acid-fast bacteria, and this led him to a detailed study of the chemistry of hydnocarpic and chaulmoogric acids. He has contributed notably to our knowledge of the constituents of *Cannabis sativa* and *C. indica* (marihuana and hashish), and he has determined the structure of some of their major physiologically active components. His wide interests are clearly exemplified by his researches on stereochemical problems, particularly of the phenomenon of restricted rotation, where his work, especially with diphenyl derivatives, and more recently with aryl olefines and arylamines, is of fundamental importance. Another indication of his versatility is to be found in his detailed studies of catalytic hydrogenation with noble-metal catalysts; the platinic oxide catalyst which he perfected is now universally employed and is known as Adams's catalyst.

While Adams's researches place him in the first rank of contemporary organic chemists, his share in inaugurating the publication of *Organic Syntheses*, and *Organic Reactions*, immensely valuable standard works of an original type, has ensured that future generations of chemists will ever remain in his debt.

During the war Adams's activities have been largely transferred to administrative spheres, where his foresight and organizing ability have enabled him to play an important part in the vast scientific effort of our American colleagues.

The HUGHES MEDAL is awarded to Professor B. F. J. SCHONLAND, F.R.S., in recognition of his important physical studies of atmospheric electricity and thunderstorms.

Schonland's main contributions to physical research have been in the field of atmospheric electricity, and have dealt particularly with the complex series of electric discharges which constitute a 'stroke' of lightning. Although the study of phenomena associated with thunderstorms could be undertaken under especially favourable natural conditions in his native South Africa, it needed enthusiasm and perseverance to overcome the many technical difficulties encountered in a country in which physical research had not yet been greatly developed, and to bring the research to such definite and illuminating conclusions.

Schonland's early work (1927, 1928) dealt with the polarity of thunderclouds; it was established that the negative was below the positive pole of the thundercloud, and that the currents flow in such a direction that they carry negative charges to the earth. Schonland also studied the importance of point discharges (from trees, etc.) in the maintenance of the earth's negative charge. He next used (1934-38) a rotating lens camera of the type devised by the late Sir Charles Boys to photograph and analyse the lightning discharge, and obtained results of great importance, for knowledge not only of the nature of the lightning discharge but of electric discharges in air in a more general sense.

Schonland has taken an active part in the study of cosmic radiation and particularly of the relation between penetrating radiation and thunderstorms; there is a reduction in the intensity of penetrating radiation when thunderclouds are overhead, and this fact provides information as to the total charge carried by penetrating radiation. The occurrence of impulses in a Geiger-Müller counter coincident with discharges in distant thunderstorms shows that some type of penetrating radiation is produced by electrical discharges during thunderstorms. This work on cosmic rays in Schonland's laboratory is of special importance, since few such systematic observations have been continued over long periods in the southern hemisphere.

Schonland has also taken a prominent part in the study of the nature of the 'atmospherics' interfering with wireless transmission, and of the part played by the ionosphere in their structure. These studies have equipped him on the technical side for various military positions he has held during the war; for these, also, his experience in the war 1914-18 as a Captain in the Royal Engineers (Signals) had given him additional equipment. In the war now ended he rose to the charge of the Army Operational Research Group with the rank of Brigadier, and later became scientific adviser to the 21st Army Group commanded by Field-Marshal Montgomery. And now Field-Marshal Smuts, as Prime Minister of the Union of South Africa, himself one of our Fellows, has claimed Dr Schonland's services as his adviser on the promotion and development of scientific research in his own country.

Last year at this time, though the outcome of the war seemed to be no longer in doubt, there was still no clear prospect of relief for science from the effort, abnormal in direction as well as in intensity, needed to ensure and to hasten victory. Now we face the new position created by the advent of victory a few months ago, with a sudden completeness beyond all prediction. We hold our Anniversary Meeting today, for the first time for six years, with our mace on the table, and all the other treasures of our long history safely returned to us by their war-time custodians, to all of whom our thanks are due. We think of the part played by scientists of the Allied Nations in the winning of the war, and with a particular pride of the contribution made by scientists of the British Empire and of this Society; and we remember that Winston Churchill accepted election to our Fellowship during the year in which he stood as leader of open resistance by the British Empire alone, to the attack which threatened to submerge the freedom of the world. If it seemed proper, I could devote all the time at my disposal to-day to a review of the various activities of this ancient Society in relation to the demands on science of a modern war, from the date of our acceptance from the Government of the responsibility for preparing the scientific section of the Central Register, at a time when the clouds of war were only gathering, down even to the present day. Some of these have been mentioned on previous occasions of this kind; and I believe that I can, in any case, use to better purpose this, my last opportunity of addressing you from this Chair, in speaking rather of matters which concern the future of the Society.

As a Society we take pride in our long history and in our steady attachment to whatever has permanent value and authority in our traditions. We may take pride no less, however, in the thought that, even among the special duties and distracting clamour of war, our Council and Fellows have found time to review our constitution, and to consider, in particular, what extensions might be desirable in the scope of elections to our Fellowship, qualitative as well as quantitative. It would seem likely that such adjustments would be needed from time to time, if a proper relation of the Society were to be maintained to the growing claims made by the natural sciences on the common stock of ability in the nation and the Empire. I find a little personal satisfaction in the thought that I, in my term as a Secretary, had a share in the initiative which led the Society in 1931 to increase its maximum annual entry to seventeen, from fifteen, at which number it had stood unchanged since, 83 years earlier, the principle of limitation was first adopted. To many this increase seemed a doubtful and even a reckless innovation, and it was accepted only after vigorous discussion and in the face of gloomy forebodings by some stalwart defenders of our traditions. So little of the foretold disaster, however, became manifest in the following six years that there was hardly any opposition to the proposal, in 1937, to add another three to the number. And now, a few months ago, after watching for eight years the effects of an election in each of twenty new Fellows, the Society has decided to raise the number again to twenty-five. My own concern with these later decisions having been limited to passive

concurrence, I should like to say now that I believe that those who promoted and the Society in accepting them have shown the right combination of enterprise and caution. During the present phase of its history, covering as yet little more than a third of the whole, election to our Fellowship has acquired among scientists, and, indeed, with a much wider constituency, a prestige which the Society will rightly wish to maintain. On the other hand, it must obviously take heed lest the rapid expansion of the number of those in this country and in the whole British Empire, who can show records of solid scientific achievement, should produce such mounting arrears of proper claims to our Fellowship, that a simple reckoning would enable a time to be foreseen when none could expect to be elected, until the days of his best service to science and to the Society were well behind him. By increasing the limit of the annual entry from fifteen in 1848, when it first became effective, to twenty-five in 1946 and till further action is taken, I cannot believe that the Society is risking a loss of prestige in seeking thus to maintain the vigour of its scientific life.

Before I addressed the Society last year the Council had already taken steps to make the way clear for a change of a different kind, by alterations of Statute approved by a postal vote of the Fellows, and made with the object of elucidating a legal position which had, in fact, existed since 1919. The Society was thus emboldened to make, last March, the innovation of including two women among the new Fellows then elected. It is hardly justifiable to assume complete unanimity among our Fellows as to the probable effect of this change in our tradition; for 10% of the large number of postal voters had expressed their unwillingness to relinquish any chance of averting it, which might still linger in the possibility of misreading our Statutes. I find myself again with the majority, in believing that this change involves no more than a perfectly normal adjustment of our practice, perhaps rather belated, to the growth in extent and distinction of women's contribution to the advancement of science by research. Perhaps, when sufficient numbers are available, some future occupant of this Chair may find himself calling upon one of our statisticians for a review of the effect of the X-chromosome on scientific output among our Fellows.

And now the scientists of the world have before them the task of readjustment which, we may hope, will mean the whole-hearted devotion of the available resources of scientific research and development to their proper and beneficent uses. It has become a commonplace that the urgent needs of war have greatly accelerated discoveries and inventions which will now promote the advancement of science and its applications in peace. Some of these scientific swords and spears will be thus immediately applicable as peaceful implements, or with only a minimum of beating and bending—radar, for example, to the safety of transport by sea and air, and all the new wealth of chemotherapeutic agents and insecticides to peace-time hygiene and agriculture. There will certainly be many others of a less direct and obvious kind—discoveries and developments arising as side issues from the urgent uses of science in war, but capable now of applications which may open new

possibilities of scientific advance for its own sake, or for a whole range of peaceful purposes. As mentioned in the Council Report, the Royal Society has recently agreed to collaborate with the Service Departments concerned, in setting up Committees to organize such peaceful uses of the special facilities for purely scientific observation and experiment as are presented by Service flying, including aerial photography, by the voyages undertaken by the ships and officers of the Royal Navy in the course of their normal duties, and by the large surplus of explosives—of the pre-atomic type, be it understood—which a great war leaves in hand. Let me make brief mention, by way of another example, of an unexpected gift to science, arising as a curious side-issue from the large-scale application in war of knowledge which science had provided. Some 30 years ago my former colleague, Dr Charles Todd, published in our *Proceedings* two papers dealing with the antigenic individuality observed, even within the limits of a single breed of chickens, when the red blood corpuscles of one bird are injected into another. This was an observation, one might think, of an interest purely theoretical, though great; but the widespread application of blood transfusion during the war, to replace blood lost by the wounded, civilians now as well as warriors, has given to phenomena of this type a practical importance. Apart from the familiar natural incompatibilities, due to the known human blood groups, it was found necessary to be alert for reactions in persons who, having had an earlier transfusion, might have acquired, by an immunity reaction, a new incompatibility to the donor's red blood corpuscles. The case of such reactions hitherto most completely studied concerns an antigenic factor which Landsteiner and his colleagues had discovered, early in the war, in the red corpuscles of the *Rhesus* monkey, and had accordingly termed *Rh*. This factor they found to be present, as a Mendelian dominant, in the corpuscles of most white people, but absent from those of a minority. So, in the slang of the subject, about 85% of people of the white races are '*Rh*-positive', while 15% are '*Rh*-negative'. Now it appears that the blood serum of an *Rh*-negative person, if he receives a transfusion of *Rh*-positive blood, acquires immune substances destructive to the 'positive' red corpuscles. In consequence, he suffers a dangerous reaction if given a second, similar transfusion. And this observation has brought to light the much more important fact that, when an *Rh*-negative woman, whose husband is *Rh*-positive, becomes pregnant by him of an *Rh*-positive child, her serum is liable therewith to acquire, and to transmit through the placenta, an antibody destructive of the child's red corpuscles, so that the offspring of such a union are prone to a high rate of mortality, before or soon after birth. Whether those who survive the infantile malady, thus produced, show a greater liability to other hereditary defects, or whether deleterious maternal antibodies of this type can be formed in relation to other kinds of cells than the red blood corpuscles, are matters on which investigation must be awaited. I mention the matter to-day as an example of the gleanings which peaceful science may expect from fields of knowledge which war has been tilling and reaping. Unless I am mistaken, the widespread use of blood transfusion has thus been

largely responsible for enabling human genetics now to explore a new category of congenital defects, due, not to the coincident presence of a detrimental gene in both parents, but to the possession by a father of one which is harmless, unless it excites an immunity response in a mother who lacks it.

Whether by following in new directions clues which have thus been discovered under the stimulus of war, or by resumption of researches which the war interrupted, it is clearly a matter of urgent importance that our scientific activities should now, as rapidly and as smoothly as possible, reacquire the character proper to peace. There are directions in which official action can accelerate a process of such outstanding significance to the position which our nation will be able to achieve and to hold in a world civilization, now so clearly entering its scientific era. We need our leaders and teachers in science back in the Universities, and the students whom they can inspire and train, as rapidly as these can be released from war-time duty and service.

A number of our leading scientists have learned much from war-time experience of organization and team-work in research, and have been freely devoting great abilities to planning and to securing proper conditions for researches by others. It is unlikely that the debt of the nation and of its allies to the work of many of these will ever be fully known, beyond the limits of certain circles. The experience of these men should help them still to serve the nation in peace, by counsel and by advocacy, when the needs of scientific reconstruction demand these. I venture to hope, however, that there will be no such demands on the time and the energies of those who should now be our leaders in research, as to keep them away from their benches and their studies, and to deprive of their inspiration the younger men who should now be their pupils and collaborators. When the world emerged from the last war, the scientists who in this and other countries were then at the height of their powers for research, who, in Newton's fine phrase about himself, were 'in the prime of their age for invention', were back in their laboratories with little delay. As a result, even in the two decades of uneasy armistice which followed, curtailed at both ends by the confusion of recovery from one war and the gathering menace of another, research for the normal purposes of peace was resumed with an astonishing promptitude, and the advance of knowledge surged forward with an imposing acceleration. Almost any man of science who can cast his mind back to the state of knowledge in his own special subject in 1919, and compare it with that which had been reached in 1939, must be impressed by the transformation. To mention an example which cannot be far, at the moment, from anyone's thoughts, consider the revolutionary changes made, between the wars, in our whole conception of the material universe, by new theories of atomic structure, with new apparatus of mathematics to deal with them, by the experimental attack on the atomic nucleus leading to transmutation of elements and, still before war's dark curtain fell again, clear evidence of atomic fission, with the release of atomic energy. As one other example, who would have predicted in 1919 that, of the vitamins and hormones then known and differentiated only by the effects of their

withdrawal and replacement, imposing ranges would have been isolated, identified and made by artificial synthesis before 1939? And now that we are emerging from another war, into what, if we scientists can do anything to prevent it, will not be just another precarious interlude before a worse disaster, we must try to ensure that the free advancement of natural knowledge, which this Society exists to promote, is able to claim again, with as little delay as possible, the full service of its natural leaders.

Another condition of the revival of scientific activity for the normal purposes of peace, seen clearly by our predecessors in 1919, was a rapid reconstruction of the international community of science. Before there had been time for the full attainment of their aim, the forces of cleavage had again begun to operate; but, as soon as it became possible once again to think of rebuilding what another war had broken, it was the first duty of the Royal Society to ensure that use was made of all that was of proven value, in the framework of international collaboration which had been constructed between the wars. Past Officers of the Society, especially Sir Arthur Schuster and Sir Henry Lyons, had taken prominent parts in the foundation and development of this organ of international collaboration. Our Foreign Secretary, Sir Henry Tizard, now coming to the end of his term of office, was early at work preparing for its revival with the Chairmen of our National Committees, in consultation with its present General Secretary, Professor Stratton, and with such representative scientists of other countries as were accessible. Their aim was to review the past achievements and potential value of this system of the International Scientific Unions, and the possible need for its extension or modification in certain directions. Our Council's Reports record the progress which has been made. While, however, the Royal Society has had a special responsibility for our national participation in this existing system, it has always welcomed any opportunity of the fullest and most friendly collaboration with any other agency for the promotion of international friendship and community of action among the scientists of different countries. The Society looks forward now to the possibility of collaborating also with any scheme or mechanism for the promotion of international relations in science, which may arise under the general Organization of the United Nations. We recognize that, through such channels, it may be possible, not only to give most valuable support to the existing Unions for international action in the various fields of scientific research, but to supplement the functions of these in many other directions in which the interests of science may yet require to be internationally organized and promoted. The Society stands ready and eager, now as ever, to work with any responsible agency for the restoration and extension of international friendship and collaboration in science.

In my address to the Society last year, I referred to the aim of building anew, and on a firm and broadening foundation, a world community in science, as 'an aim worthy of our utmost effort and devotion'. Can it be doubted now, after what has happened since I last addressed you, that upon our success in achieving that aim may well depend, not only the free progress of science henceforward, but

even the survival of civilization? I have spoken of existing and prospective mechanisms for promoting scientific intercourse between the people of different nations. We must use and develop these to the full limit of their value, but we shall still want something which no formal mechanism can restore to us. Meetings of national representatives and delegates may, indeed, do service of great importance to science, as by framing and accepting international conventions on units of scientific measurement, or on technical terminology; but no mechanism which merely brings scientists together as national representatives, no finding of formulae or passage of resolutions will do for science to-day what the world so desperately needs. If we are to achieve anything really to meet that need, we must somehow get rid of barriers which hinder the scientists of different countries from meeting simply as scientists, for the frank and informal interchange and friendly criticism of each others' observations and ideas, in complete freedom from any national inhibitions or restrictions. Before 1914 we were able to claim that science belonged thus to the world, knew no frontiers, was one and indivisible. Many of us had been cherishing the hope that the union of so much of the world in a war for the defence of freedom—freedom, we understood, for science as for all man's other activities—would have brought, with victory, a possibility of reviving this claim and restoring this ideal, which the intervening years had so shaken and obscured. Even a few weeks ago the trend of events did not appear to encourage that aspiration; but we may find, in the end, that it will suffer less from an open challenge, which all the world can see, than from a more gradual discouragement.

To all but a few scientists, as to the rest of the world, the use of the atomic bombs on Japanese cities brought the first news of a tremendous scientific and technical achievement, as well as the recognition of a new problem of overpowering importance to the world. Scientists might well take pride in it, as a triumphant verification of a purely scientific prediction. The main lines of this had been completed before the outbreak of war in 1939, by experimental and theoretical physicists of many countries. We think proudly here of the pioneer part which our own Rutherford with his pupils and associates had played in the opening of a new science of nuclear physics; but we recognize that its development was a widely international achievement. The practical realization in a little over three years of what these academic scientists had foreseen as a distant possibility, required a scientific and technical undertaking of a new order. It is unlikely that any stimulus other than the urgency of war would have sufficed to induce any national, or other, organization to embark upon such an enterprise. It is certain that, under the world conditions in which that stimulus was applied, the United States of America was the only country in the world where the project could have been undertaken. The result was a prodigy of organization and achievement, both scientific and technical; and though, in the nature of the case, America made the largest contribution even to the team of scientists, engaged in the great volume of theoretical and experimental researches still required, we may be glad to think that, on that side, the enterprise still owed much to a widely international effort. It drew into its service a large

proportion of the nuclear physicists of this country and of Canada, with others who had escaped from the clutches of the German invader in Denmark and in France, and yet others who had fled before the war from Germany, from Austria and from Italy to conditions of freedom and new opportunity in Britain and the United States.

The enthusiasm with which the world of science would normally have received the news of an event of such magnitude in scientific history was qualified by the unique conditions of its general announcement. The world at large has not been slow to grasp the tremendous implications which it may hold for the future of civilization, and the nature of the choice with which it has so dramatically faced mankind. The problems which it has raised are clearly everybody's concern. Nevertheless, and in spite of certain threats and rumblings, I believe that general opinion will allow to us men of science, in addition to our common rights as citizens, a special claim to be heard on the uses which the world is to make of this great new gift of science to mankind. General opinion, I think, would further recognize that the many scientists who have taken a direct part in this great achievement have a special right and duty to let the world know how, in the light of their intimate and expert knowledge, they view the promise and the threat which it offers to humanity. Surely they, if any, have a right to speak; and we others can welcome the firmness and the substantial unanimity with which many of them have let their opinions be known. We have a duty, indeed, to the statesmen, who are carrying this new and heavy burden of responsibility for the world's future, and who have to deal with aspects of the problem in which science is not directly concerned. It is a part of that duty, however, to keep them in touch with the general body of opinion, among the scientists of the free peoples whom they represent, so that in framing their policy they may be confident that the service required of science to make it effective will be given with enthusiasm and conviction, and not, if at all, with a reluctant acquiescence. Most, if not all of you, will have read words which our Prime Minister spoke to the Canadian Parliament, and emphasized by repeating them to our own House of Commons a week ago. This is what Mr Attlee said: 'Unless we apply to the solution of these problems a moral enthusiasm as great as that which scientists bring to their research work, then our civilization, built up over so many centuries, will surely perish.' I do not doubt that you will share my glow of gratitude for a tribute which we must try to deserve.

It is not fitting that I should discuss from this Chair matters which belong to a much wider constituency than ours, and my particular purpose to-day is to ask you to consider an aspect of this world problem, which is, beyond challenge, our special concern as men of science—the effect of present and prospective developments upon the integrity of science itself. Will any deny our claim to hold that as a sacred trust for the world, and to be alert to defend it from any danger which may seem to threaten it? I believe that we have a duty to be watchful now against a serious danger to it from the intrusion of secrecy, which we know here from long tradition and experience to be alien to the spirit of science as we have known and cherished it. I cannot claim the time which would be required to deal

adequately with such a theme. Permit me, however, to bring certain aspects of it briefly to your notice, without attempt at full or ordered discussion.

1. This danger, of course, has not newly arisen with the explosion of the first atomic bomb. We have known it long in connexion with the use of scientific research by industry, and with the relatively minor and accessory part played, till recently, by science in preparing the apparatus of war. Even in those connexions, there was a growing recognition of the detrimental effect of secrecy on the relations between the scientists concerned and the general scientific community, and a consequent effort to reduce its applications to the minimum which industrial or military opinion would accept.

2. The real and growing danger arises, however, from the new conception of war, due to the breach and consequent abandonment, in rapid succession, of conventions and restrictions which, not long ago, seemed permanent and sacred. Thus by the end of this recent war, step by step, with Germany always leading, the combatant nations had come to regard, as a proper war aim, not merely the winning of mastery over the enemy's fighting forces, but the compulsion of his surrender by indiscriminate destruction, by any means, of his people and their possessions. This principle of 'total war', as we learned too easily from our enemies to call it, having once been accepted, science found itself, no longer a mere accessory of military action, but increasingly a central agent, a direct combatant, and the provider of a limitless vista of destructive possibilities. Last year I spoke of the warning of such developments which the German V weapons had given, and the dropping of the first atomic bombs has now given to that warning a new and a sterner emphasis.

3. Preparation by our enemies for the use of science in such total war, and our own for defence against it by reprisal, have alike involved the binding of a nation's scientific effort to secrecy on a scale beyond all earlier experience. This we accepted readily, with so much else, as a necessity of war. The freedom of science, as of all that made life worth living, was at stake; if by submitting for awhile to secrecy we could help to save that freedom and to establish it for ever, we could not hesitate; but we must be watchful now against any easy assumption that that submission will be continued into peace.

4. As has now been recognized by international pronouncement, it cannot be assumed that the atomic bomb, or any contrivance using the release of atomic energy, represents the only direction, or even of necessity the most effective one, in which science could be perverted to the purposes of this 'total war', as a direct agent for the destruction of one people by another, or of dominance by the threat of it. The atomic bomb has given immediate prominence to the problem, but the world might have had to face it, even if the attempt to release atomic energy had

failed, or had never been made. The nations, in fact, have now to decide how they intend to use the powers and the resources which science stands ready to offer in growing abundance. Will they let science work again and henceforward in freedom, once more as an international community, and use what it offers for the raising of all mankind to levels of material prosperity and of culture above any that we can picture? Or will they try still to bind science to secrecy, for the competitive invention of ever more effective means of destruction, and thus hold civilization in instant peril of dissolution? It is surely our duty as men of science to help the world with our knowledge to make that decision, and to make clear our own views and intentions.

5. The danger to science from the intrusion of secrecy, against which I suggest that we need to be alert, does not arise, of course, simply from the question whether a particular technical invention shall be given away at once, or not till somebody else has made it. If policies now developing can bring about a frank and effective abandonment of all national secrecy about science, we need not, as scientists, be critical of their beginnings. Our experience so far, however, of the application of secrecy to science for military or industrial advantage, must keep us on guard. It has not, in our experience hitherto, dealt with inventions which can, once for all, be locked up or given away. The most that it has hoped to achieve has been to keep its particular employer, at each new stage, a jump ahead of his rivals. What we have now to fear is that, in default of the international agreement which we must hope and work for, national military secrecy should try to maintain, or to extend, its war-time dominance. If that were to happen, we must expect it, with its new experience of the possibilities of science in total war, to be watchful for any advance, whether fundamental or technical, whether in nuclear physics or in any other progressive field of science, which could be impounded and put under seal for warlike preparation, presumably under the name of 'security'. It is impossible to forecast how much of science might thus become involved. The release of atomic energy is yet a novelty, and we have to think what might be made of it, for good or ill, 20, 30 or 40 years ahead. I think that we, as scientists, should make it clear to the world that, if national military secrecy were allowed thus progressively to encroach upon the freedom of science, even if civilization should yet for a while escape the danger of final destruction, a terrible, possibly a mortal wound would have been inflicted on the free spirit of science itself, to the immeasurable loss of what it stands ready to offer to a wiser world.

6. I do not believe that there is any division of opinion on this issue among scientists, anywhere in the world, in so far as they are able to express it simply as scientists. We of the United Nations, in despite of all our normal traditions and instincts, were ready to submit for years to any secrecy or restriction which could help or hasten victory in the war for the world's freedom. The war has been won, and we shall not be ready to accept, as its result for science, a tightening of the chains. We have the right to expect that its freedom will be restored; and the

freedoms which we ask for science are freedom from secrecy and freedom from national barriers.

7. Secrecy as the enemy, and resistance to the attempt of authority to impose it, are no new experiences for science or for scientists. Giordano Bruno was burned at the stake and Galileo was imprisoned and threatened, because they refused to be secret about discoveries which were thought to be harmful to religion in their day; and, in a later century, there was an attempt, for a like reason, to discourage scientists by moral ostracism from telling the truth as they had seen and discovered it. Science stood firm, the world still moved, the moral stature of mankind was raised by the encounter, and organized religion gained more from it in wisdom than it lost in intellectual dominance. We do not know yet who, or how many, of Europe's scientists, in the terrible years now closing, have suffered for the scientific truths which political tyrannies desired to suppress or to distort. Unless the growth of international understanding and confidence can now prevent it, unless efforts to outlaw the abuse of science for 'total war' should succeed, science may find itself again facing an attempt to impose secrecy upon it, this time in the interests of national suspicion and rivalry, and in flat negation of its true service to mankind. If that danger should threaten, can we hope that the scientists of all the world may yet stand together against it, determined to preserve the integrity of science, to prevent its further perversion from its proper and beneficent uses, and to save civilization from misusing science for its own destruction?

Our Charter of 1663 lays down, as the object of our Society, 'promoting by the authority of experiments the sciences of natural things and of useful arts, to the glory of God the Creator, and the advantage of the human race'. Each of us may read these old words to-day in terms of his personal convictions. Freely to seek 'by the authority of experiments' and freely to proclaim the truth as science reveals it, for its own beauty and excellence and for the help, the healing and the enlargement of the means of happiness which it can bring to all mankind—does any man of science, anywhere in the world, whatever his creed or his loyalties, desire for science any aim but this? The old mandate still embodies the purpose for which the Royal Society has stood for more than 280 years, and still must stand, as a beacon to the world and as one of its centres of inspiration.

The time has come for me to retire from the Chair to which the Society called me five years ago. For none of us have the duties of these years, though different from those of normal times, been fewer or less exacting. Lack of the normal demands for social gifts or festive eloquence has caused me little sense of real deprivation. Such regret as I feel, when I look back over this interval, comes from the sense of my own inadequacy to meet the great opportunities and the heavy responsibilities for science, which a time so stirring and critical must create for one whom you have honoured by placing him here. Otherwise these years, with all their tension and anxiety, have left me many happy and grateful memories. From the Fellows as a body and members of the Councils, I have met only kindness

and consideration. I have had the crowning good fortune of serving with the same set of Officers through the whole five years. Though each of us has had too many and too urgent other duties for an ideal co-ordination of our efforts for the Society, I am sure that no happier band of brother Officers has ever worked together here. The Society is fortunate in that two of them are willing to serve yet awhile as Physical Secretary and Treasurer. Professor Hill has reached the end of a full term of 10 years devoted to the service of the Society as Biological Secretary. Though we are glad that he is obtaining freedom to reopen his proper peace-time researches, the Council have prevailed upon him to accept nomination, for another year yet, to the normally less exacting duties of the Foreign Secretaryship, from which Sir Henry Tizard is now retiring at the completion of a five years' term. The work of these four good colleagues of mine in the national interest, during the whole period of the war and in some cases for some years before its outbreak, has been largely hidden, and its effect on our scientific war effort and achievement will never be generally known or acknowledged. Here we can at least gratefully recognize their cheerful readiness to accept the additional burdens of office in the Royal Society, with all the unusual demands that these have entailed at such a time.

Then I should like to record my gratitude to Mr Griffith Davies and all the Society's staff for the friendly help which they have always afforded on the technical side of a President's duties.

You have just elected Dr Salisbury, the distinguished botanical ecologist who has also to-day received a Royal Medal, and who will enter upon his new duties as Biological Secretary with the good wishes and high hopes of us all.

And now, I am going to ask you to acclaim and to welcome my distinguished friend Sir Robert Robinson, whom you have elected to succeed me in the Chair. There are many reasons for welcoming the choice which the Society has made. Chemistry of any kind has been, hitherto, infrequently represented in this Office. Between Humphry Davy in 1820 and William Crookes in 1913 the Society elected no Chemist as its President. The great contributions to science made by both of these were to the inorganic side of Chemistry and those of Frederick Gowland Hopkins, whom we elected in 1930, dealt with its role in biological phenomena. Organic Chemistry, pure and simple, has never before been represented in this Chair, and it was the more important that, when its turn should come, the Society should be able to induce a world leader in the subject to accept election. We may well congratulate ourselves on having achieved it. I could speak long of Sir Robert Robinson's many other qualifications to be our President, and of the problems and duties lying ahead; but, lest the portrait of our Royal Founder, now above this Chair, remind you too aptly of one who was 'an unconscionable time a-dying', I am going to ask Sir Robert to come now and occupy, *de facto*, the position which we have already made his *de jure*, and to preside over the remainder of this Anniversary Meeting. I do so with the warmest good wishes and the greatest confidence.

CROONIAN LECTURE

The active and passive exchanges of inorganic ions through the surfaces of living cells and through living membranes generally

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INTRODUCTION

The main object of this paper is to discuss the large differences in concentration of individual ions between the interior of living cells and the fluids surrounding them, to bring out the point that such differences are normally, and perhaps always, brought about and also maintained by some special activity on the part of the cell, while diffusion processes are all the time tending to reduce them, and are, when maintained, expressions of *steady states*, differing in principle from equilibria by the necessity of energy being supplied for their maintenance. The pertinent facts are usually discussed under the heading 'permeability' (Wilbrandt 1938; Davson & Danielli 1943; Blinks 1942; Faraday Soc. Discussion 1937), but it will be shown below that, although permeability of the cell surface is of course a necessary corollary of the ion transport taking place, quantitative determinations of such permeabilities, in the generally accepted sense of the term, can be made only by means of isotopes and even then require special precautions and conditions which are difficult to realize and verify, because the exchanges normally taking place are largely brought about by active transport. The argument is concerned only with ions of strong electrolytes which are responsible in many cases for almost the whole of the osmotic pressure in organisms and cells, while in others they make up at least a large fraction.

Other substances will be dealt with only incidentally and for the sake of comparison. Experimental results from the most diverse sources are brought together for discussion. The experiments carried out by myself and my associates make up only a minor part of the whole, and even some of these have been published before. Forced to leave Denmark about the middle of 1944 I had to discontinue experimental work, which had already suffered severely from the difficulties encountered. I have been fortunate in finding a refuge at the Institute of Physiology in Lund, and I wish to express my deep gratitude for the kindness shown me. Everything has been done to provide facilities and papers for my work, but many recent publications have proved inaccessible in Sweden.

Although the exchanges in cells constitute the main theme, it is proposed to discuss briefly a small number of membranes composed of cells, through which a passive diffusion or an active transport of ions or both are known to take place.

I. THE EXCHANGE OF IONS THROUGH MEMBRANES

A. *Membranes allowing passive diffusion of ions only*

All membranes made up of exudation products from cells, as, for example, the cellulose walls of plant cells, the chorion of many eggs, the chitin of arthropods, etc., are only passively permeable, but the same appears to be true of a number of membranes made up of living cells or syncytia which are in the known cases flat and very thin. While non-living membranes in organisms show properties essentially like those of artificial membranes and practically constant, the properties of the living membranes may depend upon a number of conditions affecting their vitality, as, for example, lack of oxygen, presence of narcotics, etc. A few examples will be given.

(1) *Capillary endothelium (except in the central nervous system)*

Although there are definite differences in permeability between the capillaries of different organs, those of the liver and spleen being readily permeable to proteins, it can be stated generally that the capillary membrane holds back most colloids and allows crystalloids to pass. A slight and somewhat variable permeability to protein is normally present and would appear, according to the work of Fischer, Astrup & Volkert (1945), to be essential for the normal metabolism of tissue cells. Keys (1937) brought forward evidence to show that the passage of crystalloids like Ca^{++} , SO_4^- and even Na^+ is retarded when large amounts of water are filtered off from the blood during severe exercise, but this evidence is scarcely conclusive and cannot be reconciled to the fact that the smallest protein molecules are lost from the blood at the same time, while lipoid phosphorus, fatty acids and cholesterol are apparently retained (Man & Peters 1933).

There is every reason to believe that the passage of water through the endothelium, when brought about by hydrostatic pressure or by osmotic attraction, takes place in bulk and not by diffusion of single water molecules. By such passage those larger molecules which can pass through are carried with the current when this is sufficiently rapid, and very little, if any, discrimination can take place (Danielli & Stock 1944). So far as I am aware no evidence has ever been presented for any active transport on the part of the capillary endothelium, and a state of equilibrium obtains or is very closely approached between the ions on both sides of the membrane.

(2) *Glomerular epithelium*

In the kidney glomeruli it is impossible to distinguish between the capillary endothelium and the glomerular epithelium. Only a single layer of cells or a syncytium is visible. These, like the endothelium of capillaries elsewhere, are permeable to crystalloids and impermeable to colloids, and the permeability is more sharply defined. No protein, normally present in the blood, is let through,

and the concentrations of single crystalloids in the ultrafiltrate correspond very closely to those in the plasma water. The surplus driving force—the difference between glomerular blood pressure and colloid osmotic pressure—is so small that the filtration would be jeopardized even by small fractions of crystalloids held back. Along with the rapid filtration of a protein-free solution the single ions show a state of equilibrium, subject to the Donnan condition.

(3) *The blood vessels of the central nervous system*

Facts have been accumulating for several years, and certain of these have been known and utilized for quite a long time, which are conclusive in showing that the permeability of blood vessels in the central nervous system differs fundamentally from that in all other organs, while approaching that of cell surfaces, but these facts have been camouflaged by an unfortunate 'theory' postulating a 'barrier' with an indefinite localization (Lina Stern 1921) between the brain and the blood, which would prevent the exchange of certain substances, notably dyes. The 'barrier' conception is qualitative only, but the important point is that the rates of penetration of a number of substances, including ions, are very seriously slowed down, while others pass through with the greatest ease. A selection of the relevant facts is enumerated below.

(a) The 'barrier' conception is mainly based upon the fact that a dye, trypan blue (Goldmann 1913), injected into the circulation, penetrates and stains all organs outside the central nervous system, but in the brain stains only after injection into the cerebro-spinal fluid (C.S.F.). Broman has shown in a series of papers (1940, 1941a, b) that it is the capillary endothelium which is impermeable to the dye, and he consequently locates the 'barrier' to this endothelium.

(b) Just as in other organs there are comparatively large 'pericapillary' and extracellular spaces within the central nervous system. The fluid in these spaces, amounting in the brain to 20–30% of the total volume, exchanges through the capillary wall with the blood plasma, but rates of exchange differ fundamentally from those found outside the central nervous system (C.N.S.).

(c) The analyses of Wallace & Brodie (1939, 1940) and their collaborators show conclusively that the extracellular fluid in the C.N.S. comes very close to the free C.S.F. obtained from the cistern or spinal canal. This in its turn differs from a plasma ultrafiltrate in having definitely more Cl^- and Mg^+ and less K^+ (Pincus & Kramer 1923; Stary, Kral & Winternitz 1929; McCance 1936), and selective secretion or active absorption of certain ions must come into play somewhere, but has not so far been located. There is a constant slow production of C.S.F. from the ventricles, and the surplus flows off through the arachnoid villi, probably in bulk without any discrimination between the single dissolved substances, and probably also along the roots of the spinal nerves (Scheid 1941).

(d) A number of anions, viz. bromide, iodide, thiocyanate, phosphate and chloride, which in the extracellular spaces outside the C.N.S. are equalized with the

blood in a few minutes, are not even approximately equalized in the C.N.S. in 3 hr. (Wallace & Brodie 1939, 1940; Brodie & Wallace 1940; Hahn & Hevesy 1941*a*). According to Silvestri (1940) the vessels are impermeable to sulphate.

(*e*) Cations penetrate perhaps even more slowly. In experiments with radioactive sodium Hahn & Hevesy (1941*a*) found only 3 % exchange after 11 min., 12 % after 2 hr. and equilibrium after 62 hr. Experiments by Noonan, Fenn & Haegge (1940) with radioactive K showed a distinctly more rapid exchange of this ion, but still many times slower than outside the C.N.S. It follows that the electrical properties are not essential in determining the possibility of passage, as has often been maintained for dyes.

(*f*) Organic crystalloids, like sugars and urea, penetrate slowly only. Urea is found in practically the same concentration as in blood plasma, but when the blood concentration is raised to 80 or even 170 mg. % a slight rise is observed after 3-6 hr. (Cumings & Carmichael 1934). The glucose concentration in the C.S.F. or extracellular spaces of the brain is normally only half of that in the blood. It has been known, and utilized, for a long time that it is possible by massive injections of sugar into the blood to dehydrate the brain. The sugar raises the osmotic pressure of the blood, and water is attracted from the C.N.S. although not in measurable amount from other tissues into which the sugar diffuses very rapidly.

(*g*) Lipoid-soluble substances generally pass through the capillaries of the C.N.S. very easily. This is well known from the rapidity with which narcosis can be produced, e.g. by evipan intravenously, and in special experiments made by Dr Holm-Jensen, but not yet published, it was attempted to measure the relative rates of passage of chloroform, ethyl alcohol and ethyl urethane, all of which penetrate very rapidly, but in the order given. Oxygen and other gases which are fairly soluble in lipoids also penetrate easily. Bechgaard, Lohse & Vermehren (1941) studied the passage of several derivatives of sulphanilamide into the C.S.F. and found that sulphapyridine penetrates fairly easily, but sulphathiazole and sulphamethylthiazole do so much more slowly.

(*h*) Just as in cells, water penetrates very easily and even more rapidly than the lipoid-soluble substances enumerated, as shown in experiments with heavy water undertaken by Holm-Jensen. On the whole the permeability of the vessels in the C.N.S. is very reminiscent of that generally found or assumed for the cellular membrane, and it would appear that in the search for drugs which can act on this system one should be guided mainly by studies of their solubility in lipoids and not, as very generally supposed (Laurie 1943), of their electrical charge. It must be admitted that there is no proof that the endothelium of the vessels in the C.N.S. have no secretory function. Attempts to show, by injections into the blood of radioactive Cl and activity determinations on the C.S.F. from the ventricles, that Cl is secreted by the choroid have failed (Holm-Jensen), and it appears that one must look elsewhere for the secretory machinery.

A few experiments on fishes (Lundquist 1942) have shown that the peculiarities of the vascular membrane in the C.N.S. are essentially the same throughout the vertebrate series, but regarding their biological significance only guesses are possible so far.

B. *Transport of ions through membranes*

In a number of cases it can be shown that a transport of ions takes place through cells from one surface of a 'membrane' to the opposite, and when, as is often the case, a regular flow is established of a solution much more concentrated than that from which both water and ions are derived, it cannot be doubted that energy is used up in the process and (partly) converted to the osmotic force of the more concentrated solution.

(1) *The bleeding from wheat roots* (Lundegårdh 1943)

It has long been known that, when the stem of a plant is cut just above the root, water will flow out from the cut surface, and when an airtight connexion with a manometer is established pressures up to 7 atm. (tomato plants) can be recorded. Lundegårdh studied the composition of the bleeding sap exuding from the cut stems of 15-day-old wheat seedlings with the roots placed in a suitable dilute medium (Lundegårdh 1943, p. 21). He collected the sap in dry cotton-wool (figure 1) and compared it with the medium bathing the roots. Invariably the total ionic concentration of the bleeding sap, which was practically free from organic substances, greatly exceeded that of the experimental medium; e.g. sap 25 mM., experimental medium 1 mM. KNO_3 . The single ions presented to the roots in the experimental solution are of course found in the sap, but the concentration ratios differ, K and NO_3 are preferentially exuded and divalent ions to a slight extent only. A considerable fraction of the ions absorbed by the root surface from the medium is retained in the root cells and utilized for growth (NO_3^- , K^+), and this process will be discussed below (p. 157). Part of the retained ions are provisionally stored and are given off to the bleeding sap when roots are placed in distilled water for a limited period (up to 6 hr.). The mechanism of bleeding involves as the main factor an active transport of free ions against a gradient, and osmotic attraction of water takes place in consequence. The bleeding is positively correlated with the aerobic respiration, but goes on for a few hours in anaerobic conditions. The ion accumulation takes place only in the apical few centimetres of the growing roots, and the sap present here must be much more concentrated and is diluted by osmotic attraction of more water as it ascends the central vessels of the root. An active transport of water seems to be possible, as the bleeding does not stop even when the total concentration of the medium reaches 40 mM. or more, while that of the sap does not rise beyond 26–38 mM.

The cells of the living tip of the root are small and without any vacuoles. They are dividing rapidly, and just behind the tip they expand to a considerable size and become vacuolized. Even the root hairs which begin a few millimetres behind the

tip are vacuolized. The central vessel, collecting the sap, is separated by several layers of cells from the surface and takes its beginning about 1 mm. behind the tip. The energy involved in the accumulation and transport of ions is considerable, as will be shown in some detail below (p. 157), where also the mechanism suggested by Lundegårdh for the active uptake of ions will be presented and discussed.

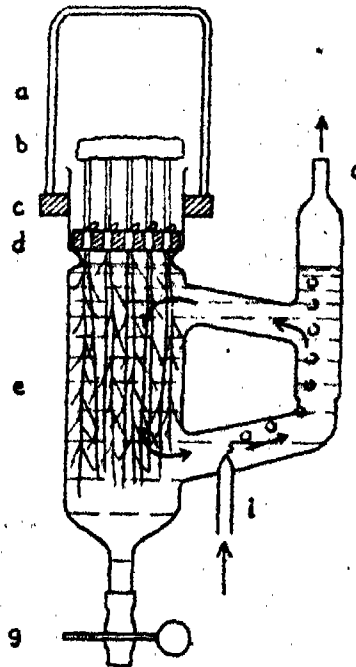


FIGURE 1. Lundegårdh's arrangement for collecting bleeding sap and determining CO_2 production of wheat roots. a, glass cover to prevent evaporation; b, cotton-wool on cut stems; d, holder with fourteen plants; e, container with 120 ml. solution; i, air inlet.

(2) *The osmotic regulation in aquatic animals*

While in the plant roots there are 'membranes' which take up and concentrate a large number of different ions at a great rate and in so doing use up energy on a large scale, in the animal kingdom there are membranes which transport only certain ions and work economically to the extent that it has not yet been found possible to measure the metabolism which must be involved. The existence of such membranes has been demonstrated in a number of aquatic animals, and in several cases they can be located anatomically. In marine teleost fishes the gills transport chloride from the blood with a Cl concentration of about 200 mM. to the sea water with a normal Cl concentration of 534 mM. (Keys 1931; Schlieper 1933). Keys & Wilmer (1932) found special cells in the gills which are taken to be responsible for this secretion, but this seems doubtful (Bevelander 1935, 1936). There must be

a transport both of Cl^- and Na^+ , and only one of them need be active. Keys thought that a solution of NaCl was actively transported, but Schlieper showed that the active transport deals with the ions, while water is attracted osmotically. The Cl transport is greatly increased when the concentration difference is reduced, but takes place only when the inside concentration is raised above that which is normal for the fish, showing that the secretion is regulated.

In starving fresh-water fishes there is a continuous loss of salts both through the urine, which cannot become salt-free, and through the gills, and the loss is made good by absorption from the surrounding very dilute solution through the gills. Normally the absorption just balances the salt loss and there is a typical steady state. To study the active absorption in detail the salt content of a fish is reduced by a more or less prolonged stay in distilled water, renewed at short intervals, in which the loss cannot be made good, and the fish is then transferred to an experimental solution generally of a single salt from which one or both ions are absorbed at a rate greatly exceeding the simultaneous loss. In such experiments it was shown (Krogh 1938a) that the goldfish (*Carassius auratus*) can absorb Cl^- against the concentration gradient from dilute solutions (millimolar or less) of NaCl , KCl , NH_4Cl and CaCl_2 and from mixtures of these salts with nitrates and iodides which are not taken up. The responsible mechanism also takes up Br^- , but neither I^- , NO_3^- nor CNS^- . Cl is absorbed (and presumably exchanged with HCO_3^-) without being accompanied by any cation from NH_4Cl and from CaCl_2 , but when a cation is absorbed simultaneously with Cl the rate is more rapid and the total quantity taken up definitely larger. An independent mechanism for cation absorption exists and will take up Na^+ from dilute NaCl , NaBr , NaHCO_3 , or from mixtures of these, and at least from the last of them the absorption is independent of a simultaneous anion absorption and is usually compensated by an excretion of NH_4^+ .

It is a very significant fact that K^+ is not absorbed at all, and from mixtures of K and Na salts the Na can be almost completely removed (reduced to concentrations below 0.1 mM.) while the K is left behind. In sufficiently dilute solutions the loss by diffusion and through the urine will exceed the absorbing power. The limiting concentration varies greatly with the species of fish and was found to be exceptionally low for the roach (*Leuciscus rutilus*), which reduced the Cl concentration of the bathing solution to 0.02 mM.

In the 'woolhanded' crab (*Eriocheir sinensis*), an animal which can penetrate far into rivers, but breeds in the sea and must be considered a very recent invader of fresh water, anion- and cation-absorbing mechanisms have been demonstrated, but they are much less selective, and it was found that the anion-absorbing mechanism would absorb, beside Cl^- and Br^- , also cyanate, thiocyanate and the very poisonous azide. All these anions are chemically nearly related, while NO_3^- , which is so powerfully absorbed by plant roots, is not absorbed at all by *Eriocheir*. The cation-absorbing mechanism of this crab does not distinguish between K and Na as does that of the true fresh-water forms so far studied. The absorbing power is very considerable, but the limiting concentration fairly high (about 0.3 mM.).

These two facts point to a relatively large loss by diffusion, and it ought to be possible in this case to measure the metabolism involved in the absorption process, when the animals again become available. In *Eriocheir* the salt-absorbing mechanisms are unknown, but must be located in the gills. In many other Arthropoda, including the fresh-water Crustacea *Astacus* and *Daphnia* and a number of fresh-water insects, ion-absorbing mechanisms have been detected which become visible by staining black when the animals are kept for some time in dilute solutions of a silver salt (AgNO_3) and then exposed to light. H. Koch (1934) showed that the absorption and accumulation of Ag^+ ions is the primary reaction, and the reduction under the influence of light secondary, and K. Schmidt-Nielsen (1941), who studied the reaction more in detail in *Astacus*, found that Ag was accumulated from solutions containing only 0.01 mM. in which Ag^+ is not precipitated by Cl. As the Ag^+ ion is chemically nearly related to Na^+ it is natural to assume that we have to do with the specific cation-absorbing mechanism which is probably clogged by the Ag. A separate anion-absorbing mechanism has been demonstrated, but not located. The reaction to Ag^+ of the ion-absorbing mechanism has been observed so far only in arthropods. It is a very important fact that the ion-absorbing mechanisms in aquatic animals are definitely regulated so as to maintain more or less exactly the normal composition and osmotic concentration of the blood and extracellular fluid of the animal. It appears natural to assume that the ion concentrations in the tissue fluids act on the cells doing work, but direct evidence has not been brought forward. Since both cations and anions are independently transported against very high gradients any true equilibrium is out of the question. When a fresh-water animal, living in a solution containing ions in very low concentration, maintains a concentration of 100 mM. or more inside its body, and this is to be done with the expenditure of a minimum of energy, the diffusion permeability of the exposed surfaces both to ions and to water must be quite small. The permeability to ions has not so far been measured in such a way that figures can be given, but the permeability for water expressed by the minute number (time taken for 1 cm² water to pass through 1 cm² at a pressure difference of 1 atm.) varies from a couple of weeks in the most permeable forms to several years, and will normally involve a very low permeability also to ions. The site of the absorption processes is in the gills, and it would seem, although no proof is available, that the flat and thin cells (or syncytia as shown for *Astacus* by R. Maluf 1940) of the gill filaments are actually doing the work. While the properties of the cell-surface film, an oriented layer of molecules or micellae a few millimicra thick, are responsible for both the passive permeability and for the active transport of ions, it is natural to assume that the energy is derived from processes taking place in the protoplasm and that quite thin cells may suffice for the absorption processes in the gills.

(3) *The ion absorption in kidney tubules*

As stated above (p. 141), the fluid entering the kidney tubule from the Bowman capsule is practically an ultrafiltrate from the blood containing all the ions in the

same relative proportions. The urine leaving the kidney is very different and is, in the fresh-water animals, extremely dilute. The chief function of the kidneys in these animals is to regulate the water balance. The blood and interstitial fluid are greatly hypertonic to the medium. Water flows in osmotically and is removed by the kidneys which reabsorb most of the salts. There are probably specific absorbing mechanisms for the separate ions (located in Amphibia to the distal section of the tubule). In mammals specific absorbing mechanisms have been demonstrated for certain ions including HCO_3^- , Cl^- , HPO_4^- , Na^+ , K^+ . Some of these have a limited effectiveness, being able, for example, to reduce the urine concentration of Na or Cl only slightly below that of the blood plasma, while the mechanism for HPO_4^- can reduce the concentration of this ion practically to 0 and does so when the HPO_4^- concentration of the plasma is normal (Pitts 1933). In a number of cases the absorption is regulated so as to maintain in the plasma a normal concentration of the ion in question.

(4) *The active ion absorption in the mammalian intestine*

In a series of papers (1936-40) M. B. Visscher and his collaborators (notably Dennis, Ingraham and Peters) studied the uptake of univalent ions in isolated loops of the dog's ileum. The general experimental method was to fill the loop with a measured volume of a mixture of two salts, both of them in a solution approximately isotonic with the blood and one of them made up with a divalent or polyvalent ion, e.g. Na_2SO_4 and NaCl . In such an experiment, lasting from $\frac{1}{2}$ to 2 hr., the Cl concentration, at the start about half that of the blood, is reduced to a very low value (absolute minimum 1.5 mM.), the fluid volume is considerably reduced and the SO_4^- concentration increased to such an extent that a very slight absorption of sulphate can be inferred. It is evident that Na^+ is taken up with the Cl^- , but the concentration of this ion is not reduced below that of the blood. In similar experiments with a divalent cation, e.g. $\text{MgCl}_2 + \text{NaCl}$, the Na^+ is reduced to a low figure, indicating active absorption against a high gradient, while Cl^- is reduced even more than the Na^+ , but cannot reach a sufficiently low level to *prove* an active absorption. Experiments were made also with a di-divalent salt along with a uni-univalent, as $\text{MgSO}_4 + \text{NaCl}$, in which case both Na^+ and Cl^- were reduced to low levels, as was to be expected, Cl somewhat more than Na. In special experiments (Peters & Visscher 1939) it was shown that ' D_2O and NaCl leave the gut, the former in the direction of, and the latter against the direction of their respective gradients at rates which approach equality when the rate of active absorption is high'. At lower rates of absorption the D_2O shift exceeds that of the NaCl . Br is absorbed along with Cl and, although it is moved with the diffusion gradient, it can be shown that not more than 10% can have been moved by diffusion. Further relevant facts are the following:

The solution in the gut always becomes slightly hypotonic.

The absolute rate of active Cl absorption increases with increasing concentration in the gut up to 134 mM.

'In no instance has the salt carried its isotonic equivalent of water with it from the gut to the blood' (Dennis & Visscher 1940b).

The divalent ions are absorbed to some extent, but, in all the experiments given, with the diffusion gradient.

Distilled water is poisonous to the interior of the gut.

To account for their experimental results, which clearly require osmotic work being done by the epithelium of the gut, the authors put forward the 'fluid circuit theory'. This theory suggests that 'in essence, the selective transport of materials against high concentration gradients is a result of the circulation of fluid through differentially permeable membranes'. A submicroscopic mosaic pattern is assumed in which one set of elements is almost impermeable to all ions and allows the passage of water only, while the other set is freely permeable to monovalent ions, but only slightly so to divalent. It is assumed further that water enters the gut through the first set of elements and washes out the content through the second. This theory will account not only qualitatively, but also quantitatively, for observations. The necessary fluid movements are not given, but in the experiment in which I have calculated them from the data given they amount to an inflow of pure water into the gut of about 150–200 ml./hr. and an outflow 26 ml./hr. higher. The 'fluid-circuit theory' is strongly at variance with all other conceptions regarding ion transport in organisms, but it is maintained (Peters & Visscher 1939) that 'none of the observed facts in connexion with "chloride secretion" by the gills of the fishes, studied by Keys (1933) and Krogh (1935), are incompatible with the hypothesis that a fluid circuit through suitable permeable membranes accounts for such processes'. This may be true, and the assumption of an active uptake of pure water from the sea into the blood of fishes through the gills simultaneously with an active secretion of NaCl solution isotonic with the blood through the same gills is no doubt conceivable, but one wonders why such active uptake could not be regulated so as to compensate by itself the osmotic loss of water and make the complicated mechanisms found (drinking of sea water and excretion of Cl through the gills) superfluous.

It should not be forgotten that the experimental conditions necessary to demonstrate the active uptake of univalent ions in the gut are highly artificial. Normally, the gut will not contain a high concentration of non-absorbable di- or polyvalent ions, and the absorption of water and salt will proceed *pari passu* with fairly small variations in concentrations and without any necessary secretion of water into the gut. Mechanisms for active transport of water are definitely known only in a relatively small number of cases and mainly from the kidney tubules (Henle loop) of mammals. In this case (and in the cloaca of birds and many insects) we have every reason to believe that water is transported as such from a solution with a high osmotic concentration (the urine) to one with a lower (the blood) without being accompanied by any ions (although ions may and will diffuse into the blood simultaneously). When in the kidneys the mechanisms for water and ion transports

are separately located (one in the loop of Henle and the others in the distal tubuli) it seems to me most natural to assume that also in the gut the water- and ion-absorbing mechanisms are separate, but acting in unison. Experiments with a number of different anions and cations will be necessary to determine the selectivity of the mechanism and to find out whether only ions of one sign are actively absorbed and those of the opposite electrostatically attracted.

II. THE EXCHANGE OF IONS BETWEEN CELLS AND THEIR SURROUNDINGS

At first sight the exchange of ions between the interior of a cell and the surrounding extracellular fluid might appear to be something fundamentally different from the exchange *through* cells making up a membrane. Considered more closely the undoubted differences become much reduced by transitional forms. In giant cells, like those of a number of algae (*Valonia*, *Halicystis*, Characeae), we have a thin sheet of protoplasm with numerous nuclei surrounding a vesicle of up to 10 ml. capacity containing sap. This does not differ very much from a membrane separating the interior of a multicellular organism from the surrounding medium. In the vegetable kingdom there are all transitions between such a cell and a young cell in a root tip containing a tiny vacuole or none at all. While the size of cells and the presence or absence of a central vacuole or vesicle does not make any serious difference with regard to the ion exchange, one has to make a rather sharp distinction between cells supported by a pressure-resistant cell wall (present in most plants and in some animal eggs) and naked cells bounded only by the surface film which shows a negligible minimum of tensile strength. In a *Nitella* cell, for example, the total inside concentration is very much higher than on the outside, and practically all the ions become concentrated although in different degrees. This involves, because the cell is freely permeable to water, a much higher osmotic pressure in the cell supported by the cellulose wall. The osmotic inflow of water goes on until the inside pressure allows it to be exactly balanced by filtration outward. The animal cells also are freely permeable to water with the consequence that the total concentration of osmotically active substances in the cell water must be just the same as outside. There is a very fair analogy between a large plant cell like *Nitella* and an animal like a *Daphnia* or chironomid both living in fresh water, and it seems worth while to bring out both the similarity and the discrepancy. In both cases there is a body wall able to support a certain hydrostatic pressure, a protoplasmic lining, continuous and undifferentiated in the case of the plant, made up of cells more or less differentiated in the animals, separating the sap or body fluid from the outside medium. Both the sap and the body fluid show osmotic concentrations far in excess of the outside medium and also a higher hydrostatic pressure, both kept up by active transport of ions from the low concentrations in the medium to the higher ones within the body, a transport which depends upon metabolic activity. In the *Nitella* cell a large number of different ions are taken up with a definite preference for K^+ , and the hydrostatic and osmotic pressures are of the same order. In the

animals only certain ions, notably Na and Cl, are actively absorbed, and the hydrostatic pressure, while giving rise to a definite turgor, is kept low by the kidney activity which again involves preferential absorption of certain ions from the 'blood' ultrafiltrate formed.

There seems to be general agreement that the surface of the cell 'protoplasm' is constituted as a special 'membrane' made up of molecules (or micellae) of a lipoid character, at most a few molecules (30–80 Å) thick and having a very low surface tension of less than 1 dyne/cm.; in many cases (perhaps normally) this is covered on the outside by adsorbed protein which may form a more or less dense network 'composed of cross-linked polypeptide chains. The size of the holes in this network will vary from place to place in the same protein molecule, and probably different proteins will show marked dissimilarities in this arrangement' (Harvey & Danielli 1938; Danielli 1936).

A. The exchange of ions in plant cells

(1) The ionic exchange in giant plant cells, the sap of which can be directly analysed

These cells are of special interest, because it is easy to show that the ions in the sap are only to a negligible extent—if at all—present in organic combination, but are free to exert their full osmotic pressure. This follows both from the very low content of organic matter present in the sap, in *Chara* and *Nitella* less than 0.3 %, of which less than 0.1 % is protein, and from the fact that a mixture of inorganic salts made up so as to correspond as closely as possible to the composition of the sap, as revealed by analysis, shows the same electrical conductivity. Osterhout (1933) tabulated the results of sap analyses on giant cells from the sea, from brackish water and from fresh water as shown in table 1.

TABLE 1. MILLIMOLAR CONCENTRATIONS OF IONS IN GIANT CELLS OF ALGAE AND THEIR NATURAL ENVIRONMENT

	sea water	<i>Valonia</i> <i>macrophysea</i>	<i>Halicystis</i>	brackish water	<i>Chara</i>	pond water	<i>Nitella</i>
Cl	580	597	603	73	225	0.9	90.8
SO ₄	36	trace?	trace	2.8	3.9	0.3	8.3
H ₂ PO ₄	0	—	—	trace	4.1	0.0002	3.6
NO ₃	0	—	—	0.005	0.4	0.55	0
Na	498	90	557	60	142	0.2	10.0
K	12	500	6.4	1.4	88	0.05	54.3
Ca	12	1.7	8	1.8	5.3	0.78	10.2
Mg	57	trace?	16.7	6.5	15.5	1.69	17.7

The marine forms are more or less globular or ellipsoid, a few centimetres long and with cell walls not particularly resistant; the *Chara* and *Nitella* cells are cylindrical, several centimetres long and 0.5 to over 1 mm. thick. They have a very resistant cellulose wall and can stand pretty high hydrostatic pressures. The total osmotic concentration in the marine genera is not much higher than that of the sea water, while the Characeae have a large surplus concentration. The figures in the

table show that almost any ion can become either concentrated in the sap or kept out of it. Cl is highly concentrated by *Nitella*, less so by *Chara*; SO_4 is concentrated by *Nitella* and definitely kept out by the marine species; H_2PO_4 is concentrated almost 2000 times by *Nitella* and even more by *Chara*; NO_3 is concentrated by *Chara* while apparently kept out completely from the sap of *Nitella*. Na is concentrated by *Chara* and *Nitella*, K by all except *Halicystis*, Ca by *Chara* and *Nitella* while kept out completely by *Valonia*. The sap of *Halicystis* differs comparatively slightly from the surrounding sea water, while *Valonia* concentrates K to a very considerable extent.

Further very suggestive results were brought to light in the very careful work of Collander (1930, 1936, 1939) on a number of Characeae, from which I give the following extracts. These plants absorb practically all cations to which they are exposed, including Li, Sr, Cs and Co. The uptake is definitely increased when they are exposed to light and often reduced by deprivation of O_2 in the dark. The total concentration of the sap increases with increasing concentration of the surrounding solution—by increasing admixture of ocean water to fresh water—in such a way that the concentration difference between the sap and the outside fluid remains approximately constant at 160–190 m.equiv. The bulk of the uptake takes place of course while the cells are growing from a microscopic bud to a length of up to 10 cm. In the case of *Chara ceratophylla* this growth took place in nature in less than 4 weeks. During this period not only is the protoplasmic membrane built up by processes in the protoplasm, but also the wall outside it is expanded by the steady addition of new cellulose molecules excreted from the protoplasm and intercalated between the old ones. It is maintained by Steward and others that in plants the active salt uptake can be realized only during growth, but Collander shows that in the Characeae the process can go on after growth has ceased and will often, and in certain species perhaps normally, lead to the final bursting of the cell which takes place with considerable violence and can be definitely heard at a distance of several metres. It may be appropriate here to give results of placing full-grown *Tolytellopsis* cells in four solutions having the same total concentration of 41 mM. but with K^+ increasing: 0.2, 1.0, 5.0 and 25 mM. In the two lower K concentrations K was gradually somewhat reduced and Na correspondingly increased, but in the two high K was taken up in excess, causing most of the cells to burst within 3 weeks.

While an uptake of ions is definitely in progress it is necessary to assume that the protoplasmic membrane is permeable to the ions absorbed, and some loss must take place by diffusion and be made good by the uptake. When a steady state is attained in the full-grown cell it is theoretically possible that it is maintained by the membrane becoming perfectly impermeable to the ions in question, but it is much more likely that some loss by diffusion continues to take place and is made good by a continued uptake. In order to settle this point it is necessary to determine the passive permeability of the membrane. Collander (1939) tried to do this by a series of ingenious experiments and succeeded in demonstrating that the passive permeability is of a very low order. He quotes a statement by Blinks to the effect that the

electric resistance of the protoplasmic membrane in *Nitella* is about 250,000 ohm/cm², which is consistent only with a very low ion permeability. He shows that lithium taken up by *Chara* cells to a concentration of 1.5 mM. is just perceptibly lost to a surrounding Li-free solution in 48 hr. According to Hoagland there is a very slow exchange of Cl in the cells of *Nitella* with Br outside. In *Chara* cells placed in a solution of 60–70 mM. KNO₃ with 2.5 mM. Ca(NO₃)₂, some Na and Cl were in all cases given off to the solution. From the quantities found in the outside fluid in four experiments after 27–90 hr. it was calculated that the cells, containing about 160 mM. Na and slightly over 200 mM. Cl, lost in 24 hr. on an average 2.5 % of the Na and only 0.3 % of the Cl. While these results do show that the permeability is very low and lower for Cl than for Na, the possibility cannot be excluded that they are vitiated by active absorption processes going on during the experiment, and it appears that the only way of determining passive permeability in cells is by the use of isotopes, chemically perfectly identical with the ions to be studied, but labelled by their radioactivity. Such determinations were undertaken on the joint initiative of Professor Collander and the writer and are published in the *Acta botanica Fennica* (Holm-Jensen, Krogh & Wartiovaara 1944). The following is a summary of the results. When mature cells have been for several weeks in a constant solution and exposed to a constant low intensity of light it is to be assumed that a steady state is reached in which the loss by diffusion of any ion is balanced by an active absorption of the same ion. When now one of the ions present is labelled by the addition of an infinitesimal amount of the radioactive isotope, without any alteration of the concentration, the protoplasm and sap of the cells will gradually become radioactive, and the activity obtained after a certain time will be a measure of the uptake of the ions in question from outside, an uptake which is assumed to be equal to the simultaneous loss. While the assumption is undoubtedly justified over prolonged periods of time and when conditions are maintained strictly constant, there is no guarantee that the active uptake proceeds at a constant rate, and rather variable results over short periods must therefore be anticipated, the more so as no precautions were taken to keep the cells in constant light during the experiments. The activities of the available isotopes of K and Na deteriorate to one-half in 12.5 and 15 hr. respectively, and even with strong preparations the experiment cannot therefore be extended beyond a few days.

Attempts to study the uptake of activity, by exposing single cells to small volumes (50 μ l.) of bathing fluid and determining the activity on 5 μ l. samples of these, failed because it turned out that large and variable fractions of the activity present disappeared almost immediately and were 'adsorbed' to the cell surface.*

* Similar adsorptions seem to be responsible for the 'induced accumulation' results obtained by C. S. Brooks (1939) in experiments with radioactive isotopes on *Nitella*. The later accumulations up to 500 mM. K and subsequent reductions in activity down to 100–200 mM. should involve a specific exchange of the labelled K or Rb ions with 'organic ions' which 'may include many so-called waste products'. It does not appear possible to discuss these views, which are at variance with our observations, while some of the ion concentrations found would no doubt cause disruption of the cells in question.

It was found possible—by centrifugation at carefully regulated speeds, for very short periods, of cells opened at one end—to obtain separate samples of sap and protoplasm and to determine the activity of each, and formulas were derived for distributing the observed diffusion resistances on the two surface membranes, separating the protoplasm from the bathing fluid and the sap respectively. While practically pure samples of sap are obtained the protoplasm samples contain a rather large admixture of sap which is allowed for by approximate determination of the true plasma volume from measurements of cell-surface areas and thickness of the cytoplasm layer. The radioactivities found in the sap (or protoplasm) are expressed in relation to the simultaneous activity of the bathing fluid and utilized for a calculation of the permeability constant P expressed in cm./hr. and corresponding to the volume in ml. of the inside fluid diffusing out through 1 sq.cm. of the protoplasmic membranes in 1 hr.

With Collander & Bärlund (1933) it is assumed that the diffusion resistance is located in the two protoplasmic membranes, while the sap and protoplasm respectively are supposed to have a uniform concentration throughout. In the experiment with isotopes on these cylindrical cells the general permeation equation

$P = \frac{v}{st} \ln \frac{C}{C-c}$ gets the following form:

$$P = 0.576 \frac{d}{t} \log_{10} \frac{C_s}{C_s - C_o \frac{a_s}{a_o}},$$

in which v is the volume, s the surface and d the diameter of the cylindrical cell in centimetres, t the time in hours, C_s and C_o the concentrations of the ion in question respectively in the sap and outside in the bathing fluid, while a_s and a_o are the corresponding activities measured. With regard to the special formula for distributing the diffusion resistance on the two protoplasmic membranes the reader is referred to the paper cited. In order to carry out the calculation it is necessary to assume that the concentration of the ion in question is the same in the protoplasm as in the sap, and single determinations made bear this out. The protoplasm was found to contain 3.9% dry substance of which 1.9 was ash. The conductivity corresponded to 252 mM. with 174 mM. K^+ . As anticipated, the results of the permeability determinations are rather variable. A summary is given in table 2.

It seems certain that *Nitella* is less permeable than *Tolypellopsis*, as one would expect from the habitat in fresh water, often very poor in inorganic substances. It appears also a fairly reliable result that K penetrates more easily than Na, as one would expect from the relative dimensions of the two ions. Most reliance is placed on the lowest figures for K, but it seems quite possible that the permeability can show wide variations and be affected by the K concentration. The figures are of the same order of magnitude as the lowest found by Collander & Bärlund (1933) for organic molecules like glucose, which are practically insoluble in lipoids. These authors could not make precise determinations for such very slowly permeating substances, but only state that P is lower than 3×10^{-5} .

TABLE 2

	date	duration hr.	number of determina- tions	C_o	C_i	$P \times 10^5$	
						range	mean
potassium							
<i>Tolypellopsis</i>	17. xi.	15-81	6	20	188	2.9-28	18 cells from water with low K
	—	15-28	3	20	150	7.5-48	
	23. ii.	21-69	4	1.4	150	1.0-4.8	
<i>Nitella</i>	—	20-68	3	0.6	90	0.4-1.0	0.8
sodium							
<i>Tolypellopsis</i>	7. xi.	83	5	26	40	0.8-1.3	1.0
	21. xi.	106-147	6	25	40	1.1-5.5	3.1
	28. ii.	47-120	4	44	50	0.7-1.9	1.1
<i>Nitella</i>	—	47-95	2	4.9	113	0.04-0.13	0.09

It is a consequence of the extremely low permeabilities for ions that the work which the cell is called upon to perform in order to maintain a steady state is also of a very low order.

The determinations of relative resistances of the two membranes showed that the resistance of the inner was at most a few per cent of the outer. There is no reason to assume any active transport through the inner membrane separating, as is supposed, two fluids with the same ionic composition.*

(2) *Plant cells generally*

The main results obtained by the study of giant plant cells are valid in principle for the large majority of plant cells enclosed in mechanically resistant, but highly permeable, walls of cellulose or similar substances. There is during growth of the cells a considerable accumulation of ions with a total concentration exceeding and most often considerably exceeding that of the surrounding solution. This excess concentration is maintained also after growth has ceased and is the source of the turgor characteristic of plant tissue. There is every reason to believe that also in the mature cells a steady state is maintained making good the diffusion losses of ions by an active uptake, although this phase of the problem is very generally overlooked (cf. review by Hoagland 1937). As in the case of the giant cells there are large differences in the affinity of the cells for certain ions. This is well brought out in a series of experiments by Collander (1937, 1941) in which a number of different

* In his book *Submikroskopische Morphologie des Protoplasmas und seiner Derivate* (1938) Frey-Wyssling states (p. 163), on the authority of Janet Plowe (1931), that the vacuolar membrane (the tonoplast) differs definitely from the outer cell membrane (plasmalemma) in containing more lipid and in being chiefly responsible for the practical impermeability for hydrophilic substances. This is strongly at variance with our result, but a perusal of Plowe's very careful and interesting papers reveals no such difference. They show both membranes to be highly tensile and elastic and to be impermeable to the dyes tested, a result which is perfectly compatible with that given here.

plants were cultivated in the same solution containing 2 mM. each of Na, K, and Rb, and it was found that K and Rb cannot apparently be distinguished by the absorbing cells, since they were present in the ash in almost equivalent concentrations, showing only minor though significant differences from one species to another. On the other hand, the absorption of Na bore no relation whatever to the two other ions, and the species could be arranged in a definite order, beginning with *Fagopyrum* and ending with *Salicornia* and *Atriplex* showing an increasing affinity for Na (figure 2).

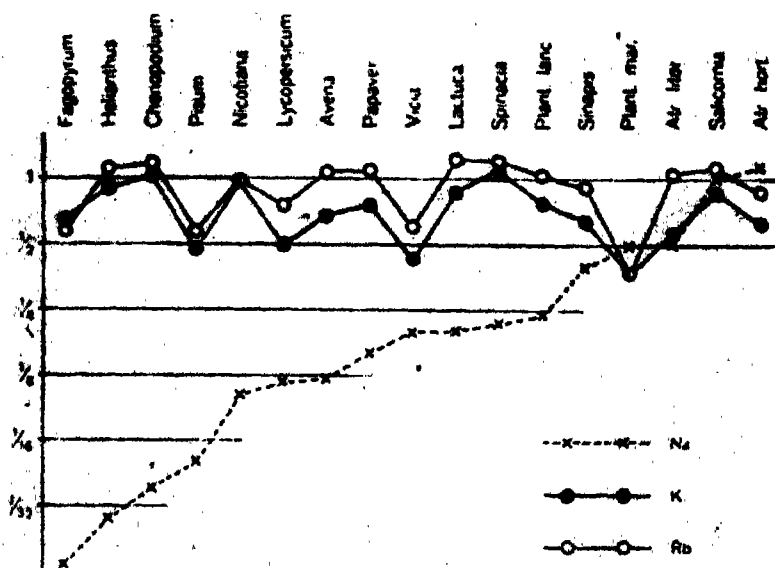


FIGURE 2. Na, K and Rb in mM./g. dry substance of a series of plants, cultivated in the same solution containing 2 mM./l. of these ions (Collander).

While the roots of plants can be surrounded by solutions of known composition the fluid coming in contact with the cells in the stems, leaves, flowers and fruits is generally unknown. As discussed above (p. 144), the bleeding sap may be relatively concentrated compared with the culture fluid or soil solution, but still far below the osmotic concentration of cells, and it is to be remembered that in normally transpiring plants it may be more or less diluted by water taken up osmotically through parts of the root system which no longer possess the power of active ion absorption. In many cases the accumulation of ions in cells can be shown to depend on the metabolic activity of cells associated with aerobic respiration and reflected in CO_2 production (Steward, papers in *Protoplasma*, 15-18, 1932-3; Steward, Berry & Broyer 1936), but it is important to note that the respiration provides the energy, and the CO_2 as such has nothing to do with the process. This is well brought out in the study by Rosenfels (1935) of the accumulation of Br from 2 mM. KBr in

Elodea leaves, in which it is shown that the uptake is brought to a standstill by lack of O_2 , although the production of CO_2 goes on, but is not definitely affected whether CO_2 is eliminated (in the dark), stationary (in light, without a supply of CO_2 from outside) or absorbed (when an outside concentration of 0.2 % CO_2 is present in light).

(3) *Ionic exchanges in roots of higher plants*

The most interesting and suggestive results are those obtained on roots. In a paper by E. Tyner (1935) the concentration of K in the culture fluid necessary for optimal growth is studied for a few plants. 0.075 mM. is found to be sufficient for buckwheat, 0.025 mM. for red clover and alfalfa. At these concentrations a K content of 1–1.5 % of the dry substance is reached, corresponding to about 100 mM. in the sap expressed from the fresh tissues. The K in this sap is at least 96 % dialysable. A number of valuable investigations by Steward and by Hoagland failed to bring out any very definite relation between the ion absorption of roots and the metabolism, while Lundegårdh (1940a) succeeded, by experiments on wheat roots, in establishing such a relation for the anion absorption, and brought out a definite working hypothesis to account for the process. It is therefore desirable to discuss Lundegårdh's results a little more closely.*

Lundegårdh worked on groups of wheat roots from genetically constant material, uniformly treated with regard to light and composition of culture fluid, so that they would grow rapidly and contain a high concentration of glucose, which is essential for the metabolism and ion transport. The plants were placed in the circulation apparatus shown in figure 1, and the gaseous metabolism determined by measuring the CO_2 output. The R.Q. is practically 1. He shows that the respiration remains constant over a suitable length of time and is the same in distilled water and in 2 mM. bicarbonate solutions of K, Na or Ca, although in these latter K will be regularly absorbed and Ca often to a slight extent excreted from the roots, while HCO_3 is never absorbed. In solutions of 2 mM. KNO_3 or $Ca(NO_3)_2$ the respiration is increased and there is a very definite relation between the respiration and the NO_3 uptake. The total respiration can be approximately expressed as $R_t = R_g + kA$ as illustrated in figures 3 and 4 from Lundegårdh (1940a, p. 316). The total respiration R_t is the sum of a fundamental respiration (R_g) and the millimoles of anion (NO_3) absorbed multiplied by a constant which in the case of NO_3 is 2 (1.8–2.2).

* Hoagland & Steward (1939, 1940) have criticized Lundegårdh's experiments and results, but it is evident from the criticism that they have not made themselves really well acquainted with the experiments criticized. They believe that Lundegårdh wishes to establish a direct connexion between the CO_2 output and the anion uptake, while Lundegårdh himself is very careful to point out that he uses the CO_2 output only as a measure of total metabolism—in cases in which special experiments have shown that the R.Q. is unity. They state that 'Lundegårdh cites no proof that submerged excised roots continue to exude salt' into the central vessel, although figures were given in 1937 showing K quantities in the exuded sap by no means negligible compared with those taken up by the roots.

Also the criticism published in 1941 by Steward & Preston is scarcely relevant. The processes in potato disks are of considerable general interest, but fundamentally different from those in roots.

A number of experiments with widely varying cation and anion absorptions conform satisfactorily to this rule. The corresponding constant for Cl^- is about 3 and for SO_4^- about 6. It is worthy of note that NO_3 largely disappears from the roots, being converted into proteins in growth processes, while the two other anions remain free. In experiments on plants cultivated in excess Ca, and therefore containing more than double the normal amount, large quantities were lost in 2 mM. KNO_3 and no Ca was absorbed from mM. $\text{Ca}(\text{NO}_3)_2$, but in spite of these differences the respiration could be approximately expressed by the formula for anion respiration.

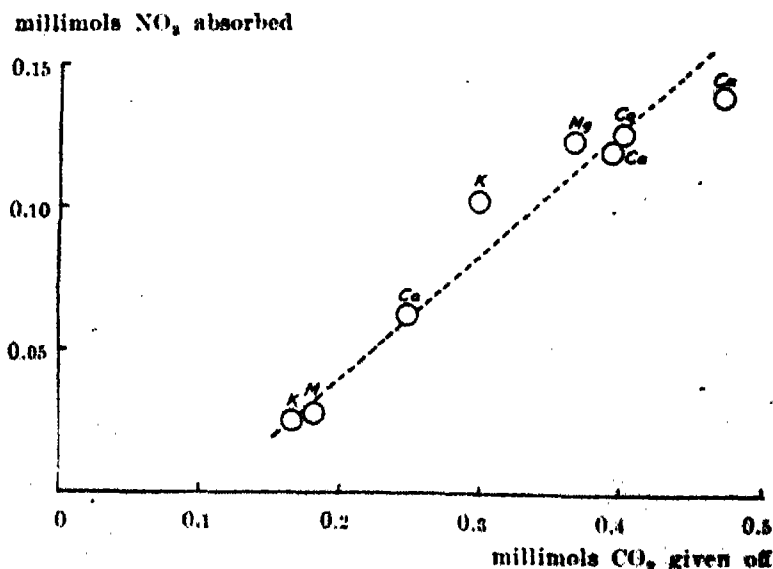


FIGURE 3. The relation between mM. NO_3 absorbed and CO_2 eliminated in experiments with nitrates of a series of cations. Anion respiration (Lundegårdh).

In very careful experiments on the roots of the halophyte *Aster tripolium*, van Eijk (1938) confirmed the existence of an additive effect of ion absorption on the respiratory metabolism, but found values for k much lower than in the case of wheat. Van Eijk also showed that a similar relation could not possibly have been detected in a number of Steward's experiments, because the respiratory intensity was limited beforehand by an insufficient access to oxygen.

The fundamental respiration calculated from the formula comes out distinctly lower (about two-thirds) than the respiration measured in distilled water or 2 mM. bicarbonate, and Lundegårdh rightly points out that this is what one should expect, because, in the absence of anions to be absorbed from the bathing fluid, the mechanism still has to deal with the anions diffusing out from the roots and by reabsorbing them maintaining a steady state. As will be discussed below, the passive ion permeability of the roots is of a high order.

Lundegårdh admits (1937) that the 'coefficient' k is not really independent of the cation present, but rises for NH_4 , Sr, Ba and H. It is not the amount of cations absorbed that is the cause of their influence on k , but their mere presence in the solution (Lundegårdh 1940a, p. 331). From the acids, especially, only small amounts of anions are absorbed, while the respiratory metabolism becomes very high. These discrepancies cannot, in my opinion, be used as valid arguments against the conception of active absorption of anions requiring definite and very considerable amounts of energy. Lundegårdh himself says (1940a, p. 322): 'It is obvious that the coefficient k does not give evidence of any stoichiometrical relations between the

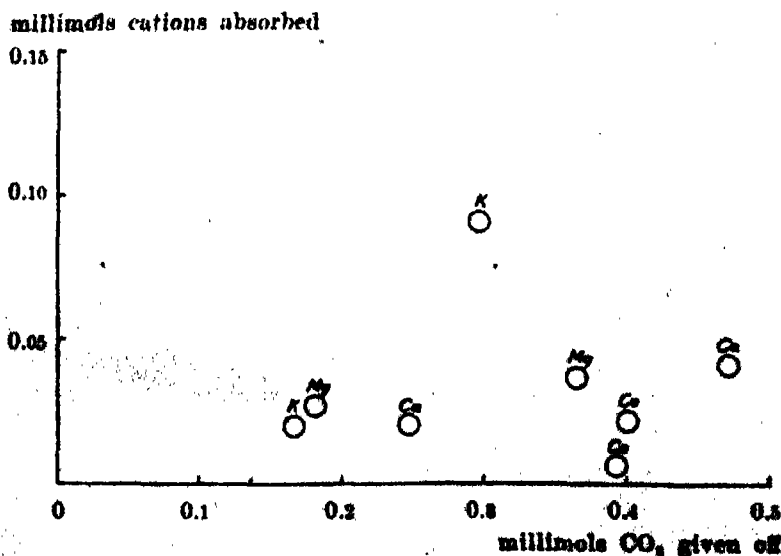


FIGURE 4. The corresponding relation between cations absorbed and CO_2 eliminated (Lundegårdh).

amount of absorbed anions and the amount of oxidized glucose. It is also evident that k is an expression of the total work performed in lifting anions from the O-level up to the I-level and accordingly is also dependent upon the manner of translocation (if the way is long or short) and upon the degree of accumulation, etc. It cannot be expected that k should show any approximate degree of constancy unless the same plants at the same stage of development and the same temperature, etc., are always used.'

Lundegårdh assumes (1937) an adsorption of anions and cations to the root surface. The cations can be combined to any negatively charged colloid particle. The anions must be bound to organic bases probably amides. A transport takes place to the accumulation 'level' where the anions are liberated by means of energy supplied by the respiration, while the corresponding cations are prevented from returning to the outside by the electric force of the anions. Experiments show that

the reaction responsible for the liberation of the anions is sensitive to cyanide which affects only slightly the fundamental respiration.

In his paper from 1940 Lundegårdh has brought together a large number of experimental results and arguments leading up to a conception of the structure and working of the protoplasmic membrane in roots of plants—cereals in particular, and especially wheat which provided the material for most of his experiments. He finds that the protoplasmic surface of the root system is normally charged electro-negatively as compared with the nutrient solution. In very dilute solutions of mineral acids the charge amounts to 150–200 mV, but in ordinary nutrient solutions it is reduced to 50–60 mV by the adsorption of cations. The maximum charge corresponds to a pH of about 3, and in acids of that concentration the surface ceases to be stable. The potentials of rye roots are lower than those of other cereals (wheat, oats and barley). Carriers of the charge are negative valencies in the surface of the protoplasm, and these are supposed to be distributed (more or less regularly) at a distance corresponding to those in millimolar acids (118A). 'Potential measurements favour the assumption that anions of neutral salts are adsorbed to the surface layer of the protoplasm by exchange. Hence the surface layer also possesses positive valencies, which in the absence of acids are saturated only by OH (and a small amount of HCO_3) ions' (p. 391).

'The surface layer of the protoplasm is conceived as being built up of long oriented molecules, in analogy with one or a few Langmuir monolayers. The main substance of the layers consists of non-dissociated molecules, and the molecules which carry valencies lie like islands among these other molecules. Positive and negative valencies are probably not carried by the same molecules, but form a sparse mosaic' (p. 392), the negative covering about 1% of the surface and the positive about 0.01%. As in Langmuir monolayers, the oriented molecules are supposed occasionally to turn round, so that the ions combined at the surface are exposed on the inside of the surface film where they can be split off either, in the case of the anions, by a process requiring the expenditure of energy or, in the case of the cations, by electrostatic forces represented by the acidity of the protoplasm. On the other hand, it is necessary to assume with Lundegårdh that the molecules carrying opposite charges are kept rigorously apart by inactive elements in the surface film. The work of Lundegårdh represents the first attempt known to me to conceive a mechanism which might be responsible for the active ion transport, and it can easily be modified so as to cover also other types of active transport, e.g. of cations or highly specialized ions, such as it is necessary to postulate in many animal transport systems. Lundegårdh points to the analogy with those polysaccharides which combine loosely with iodine. It seems possible even to picture a special type of the Lundegårdh mechanism to deal with water, if the water sphere of certain atoms can become varied. The conception is of course highly hypothetical, but it can serve as a basis for experimental work and is thereby entirely justified. A beginning was made in my laboratory in 1942, but the experiments were not published at the time and a brief account is given here.

As mentioned above, the mean distance of negatively charged elements assumed for the protoplasmic surface is arrived at from the experimental result that the surface potential corresponds to *pH* 3 and is taken as equal to the mean distance of anions in a millimolar acid solution. The reasoning leading up to this result does not appear binding, and an attempt was made to obtain a more direct estimate of the distance in question. According to Lundegårdh, all the negative valencies of the surface should be saturated with H ions when the roots are exposed to millinormal or half millinormal HCl. The H ions are held fast in distilled water, but exchanged with metal ions when the roots are exposed to a salt solution. It should therefore be possible to measure the exchange by determination of radioactivity when roots are exposed to a solution containing $^{24}\text{NaCl}$. With the assistance of Dr Holm-Jensen and utilizing radioactive sodium preparations from the cyclotron in Bohrs Institute for Theoretical Physics, I have attempted to make such measurements.

Water cultures of wheat were arranged, and when they were about a fortnight old the roots of some of the cultures were carefully measured, so that it was afterwards possible to estimate the surface and volume of a number of roots from the total length of the main roots.

In preliminary experiments, root bundles were exposed first to $\frac{1}{2}$ mM. hydrochloric acid, next to several changes of distilled water, third to a millimolar solution of radioactive NaCl, and fourth either to distilled water or inactive NaCl. Finally, the roots were cut into small pieces or either dried and directly exposed mixed with sand in a Geiger-Müller counter or ashed and afterwards exposed in the same way. Short exposures to three changes of inactive NaCl should remove all the activity from the surface, and the fact that after 1 min. exposure to 1 mM. active NaCl and 3×0.1 min. to inactive NaCl a definite activity was retained, corresponding to 0.06 mM./ml. root substance, shows that even during this short exposure a very definite amount of activity had penetrated into the root cells, had become highly concentrated, and could not be immediately removed. When the roots, after being dipped for periods from 10 to 60 sec. in 1 mM. active NaCl, were treated with distilled water a larger amount of activity was always retained, but the results were so variable that nothing like reliable determinations of the activity combined in the root surface could be obtained. There can be little doubt, however, that it exceeds, and probably greatly, the estimate of Lundegårdh.

In another series of experiments root bundles treated first with HCl and distilled water were dipped for very short periods (15–30 sec.) into an active solution of known concentration and activity. The activity of the solution was determined after the dipping and the amount taken up by the roots calculated, allowance being made for the small quantities of solution introduced and removed with the roots. Thereupon the root bundle was dipped for $\frac{1}{2}$, 1 and 1 min. into three different vessels containing a suitable quantity of an inactive 10 mM. sodium salt. These solutions were afterwards evaporated to dryness, transferred to counting dishes and the activities given off by the roots determined, again with due allowance for

the ions introduced by the quantities of fluid adhering to the roots, which were lightly pressed with filter paper between each dipping. It was assumed that in the first dipping the amount of activity combined in the surface film would be completely removed and replaced by inactive sodium, while a certain fraction should be given off from the interior. The activities obtained by dipping into the second and third solutions were assumed to be derived practically exclusively from the interior, and an estimate was made from these concerning the distribution of the activity of the first solution between the surface and the interior. The estimate is admittedly very uncertain. The total activity removed from the roots was finally compared with the quantity absorbed from the active solution. I give as an example the results obtained in an experiment with a root bundle, having a weight of 6.5 g. and an estimated total surface of 430 cm², dipped for 30 sec. in 50 ml. 10⁻⁶ molar active NaCl from which activity corresponding to 40×10^{-10} mol. was absorbed. By three washings in 10 mM. inactive NaNO₃ activities corresponding to 10.6, 7.1 and 2.1×10^{-10} mol. NaCl or a total of 20 was obtained, only one-half of the activity absorbed, showing clearly that an exchange with the interior of the roots had taken place. It was estimated that 6×10^{-10} mol. was in this case given off from the surface during the first $\frac{1}{2}$ min. dipping. This corresponds to 10¹¹ individual atoms per cm², or an average distance between them of 110 Å, in very fair agreement with Lundegårdh's estimate. This apparent confirmation does not, however, hold when the concentrations are systematically varied (table 3). Even assuming very large errors for these figures there can be no doubt about the tendency. The number of atoms held on the surface increases with the concentration of the outside solution, a result which seems difficult to reconcile to the fundamental conception of Lundegårdh.*

TABLE 3

conc. of active solutions mol.	calculated number of atoms/cm ² 10 ¹¹ ×	average distances Å
2×10^{-7}	2	225
10 ⁻⁶	8-16	112-79
10 ⁻⁵	50	45
10 ⁻⁴	200	22
10 ⁻³	700	12

The very rapid and extensive exchange with the interior of the roots is by itself interesting and significant. Here one is dealing with organs adapted for the absorption of ions from dilute solutions. These are much more permeable than ordinary cell surfaces, and their active absorption, while rapid, must be wasteful from the point of view of energy conservation. While Lundegårdh's demonstration of the anion respiration and its essential independence of the fundamental meta-

* Lundegårdh himself showed (1941, p. 575) by measurements of electrical potentials that an 'extra quantity of cations' depending on the concentration of the bathing fluid is adsorbed to the root surface and is probably only slightly dissociated.

bolism is almost certainly valid for the roots of many plants, including at least *Nicotiana* and *Aster*, it would be rash to generalize too far, and many different mechanisms may exist within the vegetable kingdom. It seems reasonably safe, however, to assume that the ion-absorbing mechanisms in plant cells are generally very incompletely specialized. There are certainly numerous cases in which certain ions are preferentially absorbed (K and NO_3 very often so), but it would appear that any ion can be taken up and to some extent concentrated in plants, provided it is not too poisonous. Many rare elements are so concentrated. The best known example is the accumulation of iodide in marine plants including both higher plants (*Zostera*) and algae, of which certain species show concentrations of many thousand times that of the sea water. In the humus of old forests and in the ash of coals from all geological periods, several, otherwise very rare, minerals are present in comparatively very high concentrations (Goldschmidt 1934). It is not possible to assert that these substances were present mainly as free ions in the sap of the plants, but it seems certain that some concentration must have taken place during the uptake through the roots, in order to account for the actual concentration found as compared with primary rocks.

Very large quantities of ions are accumulated during growth processes, and especially during budding in spring, when preformed cells expand in an incredibly short time, but there is no reason to suppose that the essential process stops short when growth ceases. The high concentrations attained have to be kept up, and although this may, in certain cases, be due to an acquired impermeability, it is much more likely to represent a steady state in which the loss by diffusion is balanced by an active uptake.

There is some reason to believe that the expansion during budding is reached in two stages, the bleeding sap supplied by the roots, from which the expanding leaves obtain their supply of water and ions, being already concentrated to a certain and perhaps considerable extent. Analyses of sap carried out just at this period, when it can be readily obtained from many plants, would provide interesting and valuable information. I have been able to find in the literature only the statement that the sap of sugar maple contains in addition to 2-3% saccharose, 0.15% ash which would correspond to about 20 mM., a substantial increase over that in the medium surrounding the roots.

B. *The exchange of ions between animal cells and their surroundings*

In the animal kingdom there is an almost endless variety of cell types. With regard to a few of these only is there any information whatever concerning their ion contents and exchange with the surrounding solution, and a ridiculously small number, comprising mammalian erythrocytes and striated muscles, have been studied from this point of view. Comprehensive generalizations cannot therefore be made beyond the point emphasized on p. 150 that in almost all animal cells the total osmotic concentration inside must be the same as that outside, in consequence

of the permeability for water and the lack of tensile strength in the cell membrane. It can only be stated that in such and such cells such and such mechanisms exist for preserving definite ionic concentrations in the cell water.

It is proposed to discuss briefly all the types of cells for which information is available, going more into detail regarding those subjected to experimental study.

All available analyses known to me indicate a definitely higher and more often much higher concentration of K inside cells than in the extracellular fluid. This K makes up a considerable and as a rule indispensable fraction of the osmotic concentration and also of the cations necessary to neutralize the anions known to be present. At least during the growth of such cells K must enter the cell against a concentration gradient. If it is combined within the cell it must be in such a way that both the osmotic and the cation concentrations are preserved.

K. H. Meyer says (1937, p. 1056): 'the electron shell of the potassium ion is almost the same as that of argon and similarly stable, so that the potassium ion cannot be masked in forming a complex with any other molecule. Only "free" potassium ions are known.'

(1) *Marine Protozoa* are in osmotic equilibrium with the surrounding sea water and swell when brought into more dilute solutions, showing thereby that the ion permeability is low compared with that for water. The most interesting form is probably *Noctiluca miliaris*, which is definitely lighter than the water in which it floats (specific gravity 1.014 against 1.024). It was shown by Goethard & Heinsius (1892) that the low specific gravity is due to a large content of NH_4Cl . The specific gravity would correspond to about 70 % of the Cl being present as the ammonium salt. The cell sap is so acid (pH 3 or lower, Gross 1934) that practically no NH_3 can be present, and we must take the protoplasmic membrane as very nearly or perhaps completely impermeable to ammonium ions. As lack of O_2 has no influence upon the specific gravity (E. B. Harvey 1917), it seems reasonable to assume that we have to do with a passive impermeability and not with an active process. The nitrogen is probably derived from the food.

(2) *Fresh-water Protozoa* and many brackish water forms are not in equilibrium with the water about them, but have a somewhat higher concentration, although generally low compared with that of fresh-water Metazoa (often corresponding to about 25 mM. NaCl). Water flows in by osmosis from the more dilute environment, and swelling is prevented by the work of the contractile vacuole (Kitching 1938) excreting pure water or more probably an extremely dilute solution. By measuring the output of the contractile vacuole, the surface of the animal and the osmotic concentration of the protoplasm, it becomes possible to determine (approximately) the permeability for water of the protoplasmic membrane, and such a computation for the form *Zoothamnium*, intensively studied by Kitching, leads to a minute number of 6 weeks, or quite a low permeability although it forces the animal to excrete its own volume of water in 25 min. In an amoeba an even lower permeability, corresponding to a minute number of 9 months, has been measured by Mast & Fowler (1935).

Experiments with 2-5 mM. cyanide, which inhibits the vacuolar contractions and causes swelling, show that the protoplasmic surface must be permeable to this ion, and it is probable that normally the internal concentration is kept up, at least partly, by active ion transport, but direct proofs are lacking. The excretion of more or less pure water from protoplasm with an osmotic concentration of 25 mM. into the vacuole is of course a vital process requiring the expenditure of energy.

(3) *Eggs of aquatic animals* will probably yield interesting results when studied systematically, but the material available to me at present is suggestive only (Bialaszewics 1929). The eggs so far studied show in almost all cases a preponderance of K over Na in the ash, but the actual figures vary greatly, and in turtle eggs there seems to be an excess of Na (Karashima, quoted from Needham (1931)). It is probably a common occurrence that marine eggs during development take up K from the sea water against the gradient, but actual observations are scarce.

Needham & Needham (1930) found that eggs of the echinid *Strongylocentrotus* absorb during the first 40 hr. of development considerable amounts of phosphate from the extremely low concentration (0.002 mM. or less) present in the sea water. The percentage amount of ash is increased tenfold simultaneously, and very probably a large fraction of this is K. As will be shown below for erythrocytes and other cells, the phosphate may enter into organic combinations and become indiffusible.

Ranzi (1930) obtained similar results on the eggs of *Sepia*.

The embryos developing in the eggs of *Nerophis* and other pipefishes show a gradual reduction in Cl content while the amniotic fluid, surrounding them within the egg, retains the Cl concentration of the sea water (Krogh 1938b). This probably means a gradual replacement of Cl⁻ by HPO₄⁻.

In fresh-water eggs instances are known of the eggs becoming completely (*Salmo* according to Gray 1920; Krogh and Ussing 1937) or almost completely (*Rana* according to Krogh, Schmidt-Nielsen & Zeuthen 1938) impermeable to water and salts, while the embryo develops on the organic material and the salts contained in the egg *ab initio*, but later there is an active uptake of salts coupled with an osmotic inflow of water.

In the case of the Japanese fresh-water fish *Oryzias latipes*, Ikeda (1937) demonstrated an active uptake of K from quite dilute solutions, down to 1 mM. and perhaps to 0.1 mM., while his results for still lower concentrations are erroneous (Krogh 1938b).

(4) *Mammalian erythrocytes* are cells which do not contain a nucleus and have therefore been assumed not to exhibit characteristic vital activities. That this view is erroneous will be made abundantly clear in the following.

The peculiar biconcave shape of erythrocytes appears to be due to surface forces, for which the suspending medium (blood plasma or serum) is at least partly responsible. In saline the corpuscles can be made to assume a spherical form without any change in volume (Ponder 1937).

The membrane responsible for the 'semipermeability' of erythrocytes appears according to Gorter & Grendel (1925) to be lipoid in character and two molecules thick. It is undoubtedly associated with proteins which may form a network on the outside. There seems to be no trustworthy evidence for any internal structure in the shape of a definite 'stroma'.

For the calculation of permeability a knowledge of cell volumes and surfaces is essential. The following average largest diameters have been assumed, viz. man 7.5μ , rabbit 7.16μ , dog 7.2μ .

E. Ponder (1935) has given the volumes and surfaces of spherical corpuscles for man as $88.4\mu^3$ and $95.7\mu^2$, and for the rabbit $61.4\mu^3$ and $75.4\mu^2$. At the normal shape the surface is larger and the figures assumed for man, rabbit and dog are given in table 4.

TABLE 4

	man	rabbit	dog
volume μ^3	88.4	61.4	61.4
surface μ^2	134	105	105

TABLE 5

	serum			erythrocytes		
	water	K	Na	water	K	Na
	g./kg.	mM./ kg. water	mM./ kg. water	g./kg.	mM./ kg. water	mM./ kg. water
dog	940	5.5	147	630	9	145
cat	940	5.94	152	630	8.7	139
rabbit	963	5.74	149	675	125*	23.5
man	940	5.9	160	680	125	36

* Average calculated from analyses by Henriques & Ørskov (1936a).

The figures available in the literature for the composition of erythrocytes are rather divergent, and this is no doubt due in a large measure to a real variability, both within the same species and even in the same individual. Some examples of this will be given below. The figures for plasma and serum are much more consistent. Table 5 shows the values I have assumed, mainly based on Abderhalden's analyses taken from Oppenheimer's *Ergänzungswerk*, I. 1, p. 21, and recalculated to mM./kg. water. These figures were calculated from the analyses without any regard to the necessary postulate of the osmotic equality of the solutions on both sides of the membrane, and it will be observed that the sums of K and Na are very nearly the same on both sides. As the alkali ions in the serum are certainly free it follows that they can, inside the erythrocytes, be in complex combinations, unable to exert the normal osmotic pressure, only to a very limited extent, if at all.

Henriques & Ørskov (1936a) found, by taking samples at two different depths from corpuscles packed by centrifugation of oxalated rabbit's blood, that corpuscles from the upper layer (that is, having a lower specific gravity) contain more K per unit volume. As the plasma content of the corpuscle samples decreases with increasing

depth, the difference, which amounts to 2–17 %, is certainly real. The immature corpuscles (reticulocytes) are chiefly massed in the upper layer, but the higher K content cannot be ascribed to these since the highest difference (17 %) was observed in a blood with only 9 % reticulocytes. The significance of this observation, which is recorded here only to emphasize the variability in corpuscle composition, will be discussed below.

(a) *The passive permeability of erythrocytes to anions.* It was discovered long ago that erythrocytes are permeable to anions and that a change in the anion composition of the plasma, e.g. in the ratio $\text{HCO}_3^-/\text{Cl}^-$, is rapidly followed by a corresponding change in the corpuscles, equilibrium being reached within a few minutes. Hedin (1897) showed in experiments with NH_4Cl and $(\text{NH}_4)_2\text{SO}_4$ that SO_4^{2-} diffuses in definitely more slowly than the monovalent anions, and Maizels (1937) gives the following relative rates of penetration into human erythrocytes, Cl being taken as unity: I^- 1.24, CNS^- 1.09, NO_3^- 1.09, Cl^- 1.00, SO_4^{2-} 0.21 and HPO_4^{2-} 0.15. There is no evidence that any of these ions is transported actively.

The permeation of phosphate was studied in considerable detail by Hevesy and his associates, utilizing the radioactive ^{32}P isotope, and it presents features of peculiar interest, illustrating as it does an important type of ion accumulation, depending upon the expenditure of energy in chemical work, but not on any active transport mechanism, because the phosphate ions are incorporated into organic compounds which are all the time broken down and rebuilt. The total amount of P in the red corpuscles is quite large. Hahn & Hevesy (1942) give the amount of acid-soluble P in rabbit erythrocytes as about 80 mg. %, but almost the whole is present in organic combination as hexose-, triose- and glycerophosphate, adenosinetriphosphate, etc.

The inorganic phosphate is, at least approximately, in equilibrium with the inorganic plasma phosphate and increases in the same ratio when phosphate is added to the plasma, although it takes several hours for an equilibrium to be reached (Hahn & Hevesy 1941a). When radioactive ^{32}P , having a half-life period of 15 days and therefore available for experiments lasting several weeks, is added as phosphate in negligible quantity to plasma it begins at once to penetrate into the corpuscles. It does not remain as free phosphate ions, but is to a large extent incorporated in the organic compounds. Hevesy & Aten (1939), and later Hahn & Hevesy (1942), showed that the rate of incorporation into large fractions of the organic phosphates is much more rapid than the rate of phosphate diffusion through the corpuscle membrane.

As the concentration of each of these substances and also of the free phosphate ions can be taken as constant, the incorporation of labelled P into the compounds means a regular breakdown to inorganic phosphate and reformation of the compounds in question, providing the opportunity for the labelled atoms to become incorporated. It is only a certain fraction of the organic P, including that in hexose phosphate and two P atoms out of three in adenosinetriphosphate, which is renewed at a rapid rate, while other compounds are renewed more slowly and some,

including the phosphatides, only to a very slight extent during the lifetime of the corpuscle. The P of these latter becomes labelled only when they are built in during the new formation of corpuscles, taking place in *in vivo* experiments of long duration. Hahn & Hevesy show that poisoning with cyanide inhibits to a certain extent the phosphorylation processes, but does not influence the rate of phosphate exchange between plasma and corpuscles.

There is reason to believe that all organic phosphoric compounds are practically unable to penetrate the erythrocyte membrane, and Hevesy & Aten have shown by direct experimentation that the permeability for hexose monophosphate is negligibly low or totally absent.

(b) *The passive permeability of erythrocytes to cations.* For a long time it has been accepted almost axiomatically that erythrocytes are impermeable to cations, because this seemed the only way to explain the large differences in cation distribution between the erythrocytes of a number of mammals and the surrounding plasma. It is clear, nevertheless, that during the formation of erythrocytes such impermeability could not possibly exist, and indications have not been lacking during the last 50 years that the impermeability postulated could not be complete even in the mature corpuscles. It is important to remember in this connexion that owing to the enormous surface area relative to the volume of corpuscles ($15,000\text{--}17,000\text{ cm.}^2/\text{cm.}^3$) even a very low permeability to cations must bring about profound changes in the cation content of erythrocytes during their lifetime if not compensated in some way. A few of these indications will be referred to.

Hedin published in 1897 very careful studies on the osmotic effect on ox corpuscles of substances added to the oxalated plasma. He determined the corpuscle volume and the freezing-point depression of the plasma resulting from such additions and found that, while the corpuscles would act as perfect osmometers against certain sugars and polyvalent alcohols, the observed figures in experiments with chlorides or nitrates of alkali metals would indicate a slight permeation into the corpuscles.

In the paper by Henriques & Ørskov quoted above, in which it was shown that lighter corpuscles of rabbits contain more K than heavier, the change in K content with age of the erythrocytes was systematically studied, and it was shown that, as the result of bleedings of 30–60 ml., the average K content of the rabbit corpuscles rises, e.g. from 305 mg. % just before the bleeding to 380 after 2 days and thereupon falls off again towards the normal value. In the dog the percentage increase in K content after bleeding is even much more pronounced and amounted to 68 % after a first bleeding (600 ml. in a dog of 21 kg.) and even to 183 % after a second (650 ml.). The absolute figures are 22.9, 38.5 and 65 mg. % or in mM./kg. water 9.3, 15.6 and 26.4. The high K content is gradually reduced, in the course of about 5 days in the rabbit, and apparently more slowly in the dog. Taken together with the observation that the heavier corpuscles in the rabbit have the lowest K content the experiments indicate a gradual leaking out of K accompanied by a slight shrinkage in volume.

The permeability of erythrocytes for cations was proved beyond any possible doubt by experimentation with radioactive isotopes of Na and K. Hevesy and his associates are mainly responsible for this work, but notable contributions were made also by American workers. The fact of the permeability comes out clearly when radio isotopes in negligible quantity are added to blood, and the washed corpuscles are found to exhibit a radioactivity which increases with time.

In some cases it is possible to utilize the results of such experiments for quantitative, although rather rough, estimates of the absolute permeability of the erythrocyte membrane.

Such an estimate must be based on the principles set out in an earlier section of this paper and there applied to the giant cells of Characeae (p. 154). For the calculation it is necessary to know the total concentration of the ion in question on both sides of the membrane, that is, in the water phase of the corpuscles and in the suspending fluid. It is necessary further to assume a steady state, in which the concentrations in question remain constant during the experimental period, so that each atom entering a corpuscle is exchanged against an identical (except for the radioactivity) atom leaving it. In some of the published cases this necessary condition was not fulfilled, and special precautions to ensure it were not taken in any of the experiments known to me. It is no wonder therefore that the results are not very precise. Nevertheless, some rather interesting conclusions appear possible.

The formula to be applied is

$$P = \frac{v}{st} \log_n \frac{C_i}{C_i - C_o(a_i/a_o)} \quad \text{or, putting } \frac{C_i}{C_i - C_o(a_i/a_o)} = q, \quad P = \frac{2.3v \log q}{s \cdot t},$$

in which v is the volume and s the surface of the corpuscle, C_i and C_o the molal concentrations of the ion in question inside and outside the cell, and a_i and a_o the corresponding radioactivities. The activity a_o should remain the same during the experimental period, while a_i corresponds to the end of the period. When v and s are measured in cm^3 and cm^2 respectively and t in hours, we get P expressed in cm./hr. and directly comparable to the figures given above for plant cells. From the volumes and surfaces given above we get for human corpuscles

$$P = 1.52 \times 10^{-4} \log q/t,$$

while for rabbit and dog corpuscles, which are taken to be alike, the factor becomes 1.34×10^{-4} .

I give as an example the determinations of Dean, Noonan, Haeghe & Fenn (1940) on corpuscles of the subject B suspended in Ringer. The results obtained by these authors are shown in table 6.

The concentrations and activities are here given per unit volume, and recalculating the figures to represent concentrations in the cell water and averages for the experimental periods I arrive at the figures given in table 7.

From these figures I calculate the permeation constant $P = 5.3 \times 10^{-8}$. A similar calculation for their subject A gives substantially the same constant, but there can

be no doubt that it is definitely too low, because the K concentration inside the cells was falling all the time, and the diffusion outwards therefore definitely in excess of the uptake of radio K measured, which was probably falling off during the experimental period, as indicated by the figures.

TABLE 6. HUMAN ERYTHROCYTES IN RINGER

time min.	hemato- crit	K concentration		relative activity		empirical curve, cell activity	slope, cell activity	dif- fusion gradient	dif- fusion coefficient $\times 10^{-3}$
		Ringer	cells	Ringer	cells				
0	5.0	5.00	(84.6)	(1000)	0	—	—	1000	—
60	5.0	5.00	84.6	1000	19	23	0.274	981	0.280
120	4.95	5.07	84.0	900	33	31	0.240	867	0.277
210	4.85	5.13	83.0	900	44	44	0.213	856	0.249
300	5.0	5.13	82.0	896	58	59	0.199	838	0.239
420	4.9	5.30	81.0	863	75	76	0.186	788	0.236
610	5.0	5.55	74.1	817	101	99	0.172	716	0.241

TABLE 7

t hr.	C_0 mM.	C_t mM.	average	final	$\log q$	$(\log q)/t \times 10^4$
			a_0	a_t		
1.0	5.0	124	950	34	0.00063	(6.3)
2.0	5.0	124	950	46	0.00081	4.1
3.5	5.1	122	950	65	0.00125	3.6
5.0	5.1	122	950	87	0.00168	3.4
7.0	5.2	121	930	112	0.00227	3.3
10.2	5.3	118	910	146	0.00314	3.1
Average						3.5

Similar experiments on rabbit erythrocytes gave, when recalculated in the same way, the value 8.2×10^{-8} , but again in this case the actual diffusion was considerably in excess of the uptake of labelled ions. An experiment on a living rabbit gave definitely higher values which, however, showed a very considerable falling off during the experimental period of 24 hr. When the last period from 6 to 24 hr. is calculated separately I find the value $P = 1.4 \times 10^{-8}$, while for the period up to 3 hr. it is possible to strike an average of 2.5×10^{-7} .

Hevesy & Hahn (1941) made a number of determinations of the rate of uptake of ^{42}K into the corpuscles of living rabbits, administering the labelled atoms by subcutaneous or intravenous injection. They state the results in tables 14–18 as the ratio of ^{42}K content of corpuscles and plasma of equal weight, from which figures it is easy to find the corresponding ratios for the concentrations in plasma water and corpuscle water respectively. When calculated from these, assuming the concentrations and also the plasma activity to remain constant, the figures for the initial periods up to 1 hr. come out very high and are, according to the authors, certainly too high. They would average about 17×10^{-7} . The relative plasma

activities appear generally to fall off slowly, and this may help to explain why also the later periods show some tendency to give values decreasing with time as shown in table 8.

The authors point out that the experiments of very long duration are perhaps vitiated by new formation of corpuscles in which the whole of the K might be replaced by labelled atoms. In my opinion this source of error cannot have any serious influence in 48 hr. experiments, because the building up of new corpuscles *ab initio* certainly takes a longer time, when not stimulated by bleeding or otherwise. Hevesy & Hahn point out that in no case has more than one-third of the corpuscle K been replaced, and they consider the possibility that only a certain fraction, less than one-half, is replaceable by exchange, like they find for muscles. In my opinion there are in the case of erythrocytes very weighty arguments, to be discussed on p. 191, against this possibility.

TABLE 8

table	<i>t</i> hr.	percentage replacement of corpuscle K	$(\log q)/t \times 10^4$	<i>P</i>
14	1.7	1.6	66	5.6×10^{-7}
	3.55	2.0	40	
	6.5	2.8	30	
15	1.33	1.8	90	
	3.5	2.5	40	
16	16.7	16	64	6.7×10^{-7}
	24.7	19	53	
	40.7	19	32	
17	18.5	14	65	7.1×10^{-7}
	39	15	34	
	48	24	61	
18	16.5	19	90	10.8×10^{-7}
	26	23	75	
	48	32	78	

In a paper by Hahn & Hevesy (1942) three *in vitro* determinations of 2 hr. duration of the uptake of labelled K into red corpuscles of the dog are given. When calculated as above these give a $P = 5.7 \times 10^{-8}$ or about 10 times higher than for the rabbit, a result not inconsistent with the low concentration of K in dog erythrocytes.

The determinations of sodium permeability carried out with ^{24}Na both by Cohn & Cohn (1939) and Hahn & Hevesy (1942) give very interesting results.

Cohn & Cohn made a considerable number of determinations lasting from 0.2 to 24 hr. on five dogs. They injected the radioactive NaCl in 10–25 ml. saline intravenously, took samples of the blood at intervals, and determined the ratio $\frac{\text{activity per ml. corpuscles}}{\text{activity per ml. plasma}}$. In some cases samples taken shortly after the injection were kept *in vitro* for a considerable period afterwards, before being centrifuged.

This made very little difference to the results, which are put together and represented by a curve drawn with $\log t$ as abscissa. From this curve I have selected four points corresponding to suitable lengths of exposure and figured out P after recalculation of their data with the results given in table 9. The average value for P becomes 1.4×10^{-5} .

TABLE 9

t hr.	percentage replacement of corpuscle Na	$C_o(a_i/a_o)$	$(\log q)/t$
0.395	10	20.1	0.163
1.67	20	39.4	0.082
5.26	34	68.1	0.107
16.65	56.5	114	0.0575

Hahn & Hevesy made a single experiment, lasting 120 min., on rabbit corpuscles. They found practically complete exchange after 15 min. which points to a very high permeability constant, of the order of 10^{-4} and certainly higher than that for K. On the blood from three separate dogs they made a number of determinations *in vitro* lasting from 0.1 to 1.4 hr., and found a very considerable exchange. When permeation constants are calculated they show a considerable decrease with time, but the values obtained after $\frac{1}{2}$ hr. or more are fairly consistent, and calculating from these I find a $P = 2 \times 10^{-5}$, definitely higher than the figure for K, 5.7×10^{-6} .

These results, if reliable, are inconsistent with the generally accepted idea of a diffusion of ions through pores which would require a definitely higher diffusion rate for K than for Na. They are definitely supported by the experiments of Davson (1940), who suspended cat erythrocytes, containing in the water phase 9 mM. K and 160 mM. Na, in 165 mM. KCl. He found that Na would diffuse out at a rate 2-5 times higher than that at which K would diffuse in. The Na permeability would increase further in a hypertonic medium, but decrease even to 0 in a hypotonic while the effects on the K permeability are in the opposite direction. The Na permeability was found to be higher in fresh blood than in that which had been stored for a day. Of course, the media in which Davson placed the cells are highly artificial and might be injurious, but it is certainly very difficult to imagine an effect *on pores* which would render them more permeable to Na than to K.

Solomon, Hald & Peters (1940) observed in human blood stored at 37°C . a considerable loss of K from the corpuscles, but an even larger uptake of Na resulting in swelling of the cells.

An interesting effect of lead upon the erythrocytes of the rabbit, rat and man was first studied *in vitro* by Ørskov (1935) and later *in vivo* by Henriques & Ørskov (1936b). Lead in very low concentration and quantity causes K to leak out from the corpuscles. Ørskov found on rabbit corpuscles 10% leakage in 64 sec. when the lead concentration was 4×10^{-6} mol. and in 29 min. at a concentration of 2×10^{-7} mol. The permeability to Na apparently remained substantially unaltered, as the corpuscles showed a definite shrinkage corresponding to the loss of K. Ørskov

observed K losses up to 85 % in 20 min., and a loss of 77 % in 1 hr. was recorded by Henriques & Ørskov after administering 20 mg. Pb to a rabbit. The enormous increase in K permeability without any detectable influence on the Na permeability is extremely difficult to reconcile with the pore permeability theory.

The fact that so much K can be rapidly lost by corpuscles exposed to a substance which may be supposed to act practically exclusively on the membrane shows clearly that this K must be completely exchangeable, existing as free ions within the corpuscle and can neither be in complex combination nor enclosed within impermeable structures inside the corpuscle.

The higher permeability constants found for Na as compared with K, taken together with the results of Davson, Solomon *et al.* and Ørskov & Henriques just referred to, seem to be incompatible with a pore theory for permeability, as there is no doubt that Na^+ carries a larger 'hydrosphere' than K^+ , diffuses more slowly and should require larger pores.

On the other hand, the results can be brought into harmony with the fundamental conceptions of Lundegårdh concerning the mechanisms for ionic exchange. We have then to assume a larger number of micellae (molecules) possessing special affinity for Na than for K in the erythrocyte surface—or alternatively, the Na-binding micellae turning round at shorter intervals of time.

(c) *The active transport of cations.* When cations inside the erythrocytes exert normal osmotic pressure and are free to move in the whole of the corpuscle water, when, further, the concentrations of the single ions differ more from the corresponding concentrations outside than can be explained by a Donnan effect, and when finally the erythrocyte membrane is definitely, even if slightly, permeable to these ions, the concentration differences can be produced and *maintained* only by some active transport: we are face to face with a steady state and not with any equilibrium. Several investigators have demonstrated the existence of an active transport.

The first experiments of this kind seem to be those of Harris (1940) in a paper which is inaccessible to me at the time of writing. According to the review by Blinks (1942), Harris found that erythrocytes which lost K by storage at 2–5° C would take up the ion again at 37° C, but not markedly at 25° C. No recovery would take place in the presence of 20 mM. NaF, which, indeed, caused a loss of K even at 37° C (contrary to the statement of Davson & Danielli 1938).

The results of Harris were confirmed and extended in a paper by Danowski (1941). He found that in human blood kept at 7° C the serum K will rise at an approximately constant rate of 0.2 m.equiv./l./hr. for up to 48 hr. without any transfer of water. As the cell volume is about 50 % the cells must lose K at about the same rate. In further experiments blood was incubated at 37° C, resulting in a striking drop in serum K of about 1 m.equiv./l. during the first 5 hr. (corresponding to an uptake into the corpuscles of 1.25×10^{-11} mol. K/cm²/hr.), but thereafter the serum K would rise, and simultaneously the corpuscles would swell as shown by an increase in cell volume and serum protein concentration. This latter

fact indicates in my opinion an unchecked inflow of Na into the corpuscles in excess of the K loss, but is considered by Danowski as indicating breakdown of phosphate esters and glucose. It turned out that the active transfer of ions was bound up with glycolysis. After about 5 hr. at 37° C the glycolysis, normally taking place at the rate of 15 mg. %/hr., was complete, and addition of glucose at this or a later stage would stop the loss of K and bring about a renewed uptake. Addition of fluoride (6–12 m.equiv.) would retard glycolysis and bring about a rapid loss of K from the corpuscles (0.7 m.equiv./l./hr.) without any change in cell volume.

Danowski's main result was confirmed on heparinized rabbit's blood in an (unpublished) experiment in my laboratory. The corpuscles were centrifuged down and samples of 1.8 ml. (of which 0.2 ml. was extracellular) were added: A to 4 ml. plasma, B to 4 ml. Tyrode solution without K or glucose, and C to Tyrode with K and glucose.

The sample B kept at 38° C hemolysed so rapidly that no precise determinations were obtained, but there was a rapid loss of K from the corpuscles.

The A sample was placed at 7° C for 1.85 hr., resulting in a loss of 8 μ .equiv. K, while the concentration in the plasma rose from 6.2 to 8.1 mM. During the same period the cations in the plasma showed a decrease of 3 μ .equiv. corresponding to 11 μ .equiv. Na entering the corpuscles. The difference of 3 μ .equiv. is inside the limits of error, but that some Na has replaced the K in the corpuscles seems certain. The glucose concentration fell from 149 to 134 mg. %, corresponding to a catabolization of 0.63 mg.

In a second period of 1.7 hr. the remainder of the sample, 2.5 ml. plasma + all the corpuscles, was kept at 38° C after an addition of glucose raising the concentration to 348 mg. %. 6.7 μ .equiv. K were taken into the corpuscles (corresponding to 1.5×10^{-10} mol./cm²/hr.), while 4.37 mg. glucose were used up. The observed cation reduction in plasma amounts to 5 μ .equiv., but the calculated elimination from the corpuscles of 1.7 μ .equiv. Na is very uncertain.

The corpuscles in Tyrode at 38° C (sample C) took up 3.7 μ .equiv. K in 2.2 hr., reducing the concentration from 7.3 mM. to 6.4 using up 1.9 mg. glucose, but in the second 2.1 hr. period in the same conditions they lost the same quantity along with a glucose catabolization of 1.36 mg. According to the experience gained in our laboratory rabbit corpuscles remain functionally normal in salt solutions only for a very limited period.

In a paper by Hegnauer (1943), known to me only in abstract, it is shown that in rats a diet containing only 0.046% K decreases the K and Cl content of the plasma and greatly decreases the K content of the erythrocytes and muscles, while their Na content is correspondingly increased. Repeated injections of desoxycorticosterone acetate given during the period of low K diet restore the K content of the erythrocytes, but not of the muscles, to the normal level and must, according to my general views, stimulate the power of active ion transport.

The experiments now described, and especially those of Danowski, which were repeated and varied so as to remove any possible doubt, establish the power of the

erythrocyte membrane of effecting an active transfer of ions. It appears worth while in the light of this knowledge to reinterpret certain earlier experimental results.

Henriques & Ørskov (1936*a*) anemized rabbits by subcutaneous injections of phenylhydrazine ($\frac{1}{2}$ ml. 1% solution/kg./day for 5 days). They summarize the results as follows: The injections cause a very large fall in hemoglobin, and after a few days all or almost all the corpuscles are reticulocytes. These are large, poor in Hb and in one experiment also poor in K. This must mean increased permeability which returns to normal when the injections are stopped, and at the same time the K content of the corpuscles rises considerably (in one case 20% above the initial value). In the writer's opinion the new corpuscles on maturing have taken up K from the plasma.

In another series of experiments the authors injected distilled water into the circulation (110 ml. in 85 min. to a rabbit of 2.4 kg.). This caused a slight hemolysis, a drop in the K content of the 'intact' corpuscles from 322 to 273 mg. % after 2 hr., followed by a very rapid rise reaching 350 mg. % $3\frac{1}{2}$ hr. later.

In this short time the new formation of corpuscles is negligible and there can be practically no doubt that the slightly damaged corpuscles have recovered and taken up K.

In the experiments on the lead effect *in vivo* (Henriques & Ørskov 1936*b*) the same authors observed, as mentioned above, a very rapid fall in the K content to 80% in 35 min. with a corresponding shrinkage in volume. Calculating the average volume and K content of a single corpuscle from the figures given by the authors, I found the results shown in table 10. The figures in the last column were calculated on the assumption of a constant amount of dry substances of $25.6\mu^3$ in the corpuscle. The low values found after the lead injection shows, when it is remembered that the total cation concentration must be at least 150 mM., that a considerable amount of Na must have diffused in during the first hour to be eliminated again later.

TABLE 10

<i>t</i> hr.	number per ml. $\times 10^9$	corpuscle vol. $\text{cm}^3 \times 10^{-12}$	K content mg. $\times 10^{-12}$	K in corpuscle water, mM.
0	5.91	67.6	229	139
0.17	lead injection, 20 mg.		—	—
1.0	5.27	46.6	35	43
2.6	3.26	63.0	38.5	26
4.5	3.37	56.6	37.5	31
23.2	2.23	73.8	159	84
47.2	2.23	60.0	164	122
71.2	2.49	66.0	196	124

The rise in K taking place from 4.5 to 23.2 hr. cannot possibly be due to any new formation of corpuscles, which is insignificant, to judge from the small number of reticulocytes (6‰), but must be brought about by absorption of K from the plasma.

When cation concentrations in the corpuscle water are maintained by active transport it appears necessary to postulate some mechanism regulating the transport. If K could be poured continuously into the corpuscles of man or the rabbit from the low concentration found in the plasma we should expect the cells to go on swelling. Experiments to test this point and find the regulating mechanisms were planned in my laboratory, but could not be completed. We succeeded only in obtaining one negative result. It was assumed that the corpuscle volume might furnish the regulator: that shrinkage of the corpuscle might induce K uptake, while swelling might stop the active process and allow some K to diffuse out until the normal size was restored.

Experiments were made therefore on four blood samples of 3.8 ml. each with the same initial, slightly increased, K content (7.5 mM.) in the plasma, to which were added respectively 0.2 ml. water, 0.2 ml. 0.9%, 0.2 ml. 1.35% and 0.2 ml. 1.8% NaCl. The hematocrit showed the differences in corpuscle volume to be expected, but there was no significant difference in the uptake of K into the corpuscles during a 2 hr. experimental period, except that it was slightly higher in the normal sample.

The problem of the regulation must be left open for the present.

It is not possible from the data recorded to choose between the possibilities of an active K uptake or an active Na elimination, but, as Hegnauer's experiments (p. 174) show the mechanism to be different from that in muscles and, as will be shown below, an active elimination of Na is the most likely alternative in the latter case, an active K uptake is perhaps the most probable for the erythrocytes of man and the rabbit. The observations of Henriques & Ørskov suggest that the power of such uptake is at its maximum in the young erythrocytes and gradually wanes with increasing age. It would appear, according to the observations on dogs by the same authors, that even in the young corpuscles of these animals, which differ so little in ionic composition from the plasma, there exists a definite power of active potassium uptake.

(5) *Ion transport in the chorion membrane of birds' eggs*

The exchange of K and Na has been studied on the chorion membrane from hens' eggs incubated 9 days (Krogh 1943). An abridged account of the results will be given here. The membrane consists of an outer (ectodermal) and an inner (entodermal) epithelial layer of cells, and between them a loose network of primitive branched connective tissue cells with a continuous and large extracellular space.

By measurements and countings on sections (5μ thick) the membrane thickness was found to be 156μ of which 9μ is the ectodermal and 1μ the entodermal layer. 1 g. fresh membrane has an area of 65 cm^2 , contains about 10^9 cells, of which 40% are in the outer, 9% in the inner layer and 51% between them. Of these latter 8% are estimated as belonging to blood vessels, but this estimate is admittedly very rough. Owing to the irregularities of the membrane the total epithelial surface of 1 g. exceeds 4×65 and is estimated at 320 cm^2 . The space between the two layers

is about 0.94 ml., but the cells make up only about 0.3 ml. according to determinations of extracellular space. If the 4×10^8 connective tissue cells are taken to be spheres the radius of each works out at 5.5μ and the total surface at 1600 cm^2 . The cells are very far from being spheres, and 3200 cm^2 is a more probable estimate, making the epithelial surface just 10% of the total, while the volumes are in the ratio $65/365 = 18\%$ of the total. The connective tissue cells are thus quantitatively preponderant while the epithelial layers make up a fraction far from negligible.

The amniotic fluid normally bathing this membrane contains, according to our analyses, reducing substances corresponding to 40–50 mg. % glucose, 7 mM. K and a total of about 130 mM. cations. The experiments were carried out in Tyrode solution with a cation concentration of about 150 mM.

All experiments were made on membranes cut into small pieces of about 20 mm^2 so as to ensure a fairly rapid renewal from the sides of the fluid between the connective tissue cells. The membranes were suspended in the experimental solutions in suitable containers, packed by gentle centrifuging (500–1500 rev./min. for 3 min.) and weighed after sucking off the supernatant fluid. Repeated weighings during several hours always show a shrinkage of the material. Usually, when conditions are nearly physiological regarding concentration of K, glucose and O_2 , this affects practically only the extracellular space, but in some cases also the cells themselves.

Membranes placed in K- and glucose-free Tyrode at 38°C lose K and take up Na, and the K concentration of the cell water can become reduced to 50 mM. with an extracellular concentration of 1.6 mM. When such membranes are placed in Tyrode containing K at any higher concentration (from 3 to 15 mM.) and glucose they absorb K at rates up to $9\mu\text{equiv./g./hr.}$ and may reduce the outside concentration to 1 mM., at the same time eliminating Na against the concentration gradient. The uptake depends upon the presence of a suitable source of energy which can be either glucose or lactic acid. The amount of glucose broken down at 38°C is much higher than can be accounted for as completely catabolized by the simultaneous O_2 absorption, but no quantitative relation between the glucose consumption of 0.3–1 mg./g./hr. and the K absorption has been found. Even in the presence of glucose, K is given off to a K-free solution, but a steady state can be attained at outside concentrations below 1 mM.

At low temperatures ($3\text{--}7^\circ\text{C}$) the membranes lose K at a rate of $1\mu\text{equiv.}^*/\text{g./hr.}$ or less, practically irrespective of the outside concentration, and the sugar consumption is reduced to less than 0.1 mg./g./hr.

Without oxygen at 38°C the sugar consumption is greatly increased (to 2 mg./g./hr. or more) and the K uptake was in several experiments found to be retarded, but in one case remained normal. The processes studied must take place mainly in the connective tissue cells, but whether or to what extent the epithelial cells may be involved is uncertain. The active process may be either a driving out of Na or an uptake of K or perhaps both.

(6) *Cross-striated muscles in frogs and mammals*

(a) *Structure.* It is not necessary to discuss the structure of muscles in any detail. The osmotically active elements are the cells or fibres which vary rather widely in length and diameter, but can for our purpose be taken as cylinders (although many are somewhat conical) of infinite length and having diameters between 50 and 200μ . Dr Buchthal advises me that 100μ is a fair average for the diameter in all the cases studied. There are indications, to be discussed below, that diffusion within the fibre is inhibited, and it appears possible that the double refractive elements, mainly responsible for the contractility and making up according to Buchthal about 60 % of the whole, are separated from the rest by a membrane of very low permeability.

In the interspaces between muscle fibres we find the blood vessels making up according to their state of contraction from less than 1 up to 10 % of the muscle volume, and the rest of this extracellular space is filled with an exudate from the blood having practically the same ionic composition. The total extracellular space in muscles is fairly small. It is usually identified with the chloride space, the fibres being considered normally chloride free and chloride impermeable. This identification is probably not quite correct, a small fraction of the chloride being inside the fibres. The extracellular space is in frog muscles slightly less than 13 % of the fresh weight and of about the same magnitude in mammals.*

(b) *The dissolved substances in frog muscles.* Hill & Kupalov (1930) made careful comparisons between the osmotic concentrations as revealed by vapour-pressure determinations of frog blood and muscles and the molal concentrations of single ions and other solutes as determined by analysis.

They found the vapour pressures of blood in osmotic equilibrium with the muscles equal to that of 0.726 g. NaCl in 100 g. water; they found the muscles to contain 800 g. water per kg., of which at most 30 g. could be 'bound', in the sense that it was non-solvent to added substances. Utilizing the available analyses they calculated the millimolal and equivalent concentrations of the single cations of muscles as shown in table 11 and the anions as shown in table 12.

The sum of molal concentrations of cations and anions is $161 + 78 = 239$, while that of 0.726 % NaCl is 248. The deficit is almost exactly made up by creatine. The deficit in anions is assumed to be made up by protein existing within the muscle as the ionized alkali-protein salt and having a negligible molal concentration.

The essential point in these calculations, as pointed out by the authors, is that to account for the observed osmotic pressure of frog muscle it is necessary to assume that all its known soluble constituents are in fact dissolved in the observed 'free'

* The extracellular space is to a certain extent (about 3 % of the muscle volume according to Manery, Danielson & Hastings 1938) taken up by connective tissue dry substance and is therefore slightly larger than assumed in the following (15.6 instead of 13 %). It is confirmed that the watery phase of connective tissue comes very near to serum ultrafiltrate and especially that it contains Na and K in nearly the same proportions although with slightly less Na. The influence upon the determinations used in the following is slight only.

water of the muscle. 'There is no latitude for supposing, for example, that an appreciable part of the K is combined, and not in free solution as a separate dissolved ion.'

Similar computations were made more recently by Boyle & Conway (1941). They took the further necessary step of distinguishing between the extracellular and the 'fibre water'. Taking all the water to be 'free', the 800 g. water in 1 kg. muscle is distributed with 130 g. extracellular and 670 g. intracellular. The results of recalculating their figures for frog plasma to plasma water are given in table 13. The sum of cations works out as 161.5 mM. and the osmotically active total as 223 mM. for the plasma water and 218 mM. for the fibre water. All the values given refer to muscles at complete rest. In working muscles important changes occur which it will be convenient to discuss at a later stage. For some mammalian muscles similar, but less complete, data for the resting condition can be made out. They too will be briefly referred to in the following in connexion with the experiments discussed.

TABLE 11

	K	Na	Ca	Mg	total
mM.	109	31	7	14	161
m.equiv.	109	31	14	28	182

TABLE 12

	Cl	HCO ³	lactate	phosphate		total
				inorganic	organic	
mM.	19	7	3	14	35	78
m.equiv.	19	7	3	26	69	124

TABLE 13

	muscle mM./kg.	plasma water mM./l.	fibre water mM./l.
K	84.6	2.6	126
Na	23.9	103.8	15.5
Ca	2.5	2.1	3.3
Mg	11.3	1.25	16.7
carnosine	11.0	—	16.4
hexose monophosphoric acid	1.9	—	2.8
phosphagen	25.8	—	38.5
adenosine triphosphate	3.2	—	4.8
protein	1.6	0.6	2.3
chloride	10.5	74.3	1.2
lactate	0.5	3.45	0.5
bicarbonate	3.6	25.85	0.4
phosphate	0.4	3.25	0*
sulphate	0.3	2.0	0
glucose	0.5	4.1	0

* As the fibre is permeable to phosphate it seems certain that the phosphate concentration in the fibre water must be at least of the same magnitude as outside.

(c) *The passive ion permeability of frog muscle fibres.* Although the contribution of Boyle & Conway (1941) is one of the latest available it will be convenient first to present their views. They treat the muscle fibres of the frog sartorius as a physico-chemical system pure and simple, postulate their permeability to the smallest cations and anions, viz. K and Cl, with impermeability to Na and to the organic phosphates inside the cells, deduce the theoretical equations to describe the resulting conditions, and 'predict with surprising accuracy the mean changes in potassium, in the smaller permeating anions (e.g. chloride) and in the cell volume—the latter to within a few per cent with changes in fibre water up to 100%', resulting from soaking the muscles in series of solutions with increasing K content from 12 up to 100 mM. or more.

They describe two main series of experiments. In one they have increased the total concentration of the soaking fluid by adding increasing amounts of KCl to an otherwise constant Ringer fluid of Barkan type, resulting in an increasing accumulation and concentration of K and Cl within the cells, the K concentration always being greatly in excess of the outside, while the cell volume remains substantially unaltered.

In the other series they have kept the outside concentration constant, but increasingly substituted K for Na, resulting in a practically constant K concentration inside—always in excess of the outside—and a large accumulation by uptake of K and Cl with water, resulting in swelling of the fibres up to above double the normal volume. It is perfectly true that in both cases the agreement, at all K concentrations above 12 mM., of the experimental results with predictions from the theory justify the postulates made, and we have to admit that fibres in these experiments are in fact permeable to K and Cl and impermeable to Na. The importance of this demonstration can scarcely be overestimated.

When, however, the authors go on to build upon it a general theory for accumulation of K in cells it is not possible to follow them. They assume the permeability to be governed by the size of pores in the fibre 'membrane', and calculating relative ion diameters from their determined velocities under the gradient of 1 V/cm. (0.5 V/cm. for divalent ions) they arrive at the figures shown in table 14. This would place several important ions (Ca, Mg and HPO_4) among those which cannot possibly enter the fibres, although they certainly do so during the growth and at least HPO_4 also later as shown below.

TABLE 14

cations		anions	
H	0.20	OH	0.37
Rb	0.96	Br	0.96
Cs	1.00	I	0.97
NH_4	1.00	Cl	0.98
K	1.00	NO_3	1.04
Na	1.49	CH_3COO	1.84
Li	1.95	SO_4	1.89
Ca	2.51	HPO_4	2.29
Mg	2.84		

From the point of view of an old and old-fashioned physiologist it is perhaps a little remarkable that the muscles kept in those solutions which are most nearly physiological with respect to K content (< 12 mM.) behave differently and are evidently permeable to Na which enters slowly while K is lost. This behaviour is explained in the paper by the plausible assumption that the artificial solutions cannot fully replace the genuine plasma and are 'improved' by the addition of some extra K. Excess K is, however, known to be rather poisonous, and one begins to wonder whether the muscles kept for 2 hr. at room temperature or 24 hr. at 3°C in the high K solutions have been able to retain their normal excitability.

I have found no excitability tests in the paper by Boyle & Conway, and Drs Buchthal and Folkow very kindly undertook to supply this control. Their experiments were made on m. semitendinosus from *Rana temporaria*. The normal Ringer contained 2.67 mM. K and more concentrated solutions were made both by adding KCl and by substitution of K for Na. Muscles kept in the normal Ringer for 24 hr. at low temperature retained their excitability unaltered as measured by the threshold stimulus, but even $1\frac{1}{2}$ K (4 mM.) raised the threshold slightly. At 4 K (10.7 mM.) the excitability was considerably reduced and at 8 K completely and irreversibly lost. In experiments at room temperature (20°C) half an hour at 10.7 mM. K had no ill effect, but 20 min. at 21.4 mM. both in isotonic and in hypotonic solution reduced the excitability which could be restored to normal in 20 min. in normal Ringer. 20 min. in 41.7 mM. K completely abolished the excitability, but the muscle from hypertonic K Ringer could be fully restored in 20 min., that from isotonic only partly so. 20 min. in 64 mM. K irreversibly abolished the excitability both in isotonic and in hypertonic Ringer.*

The muscles studied by Boyle & Conway were therefore certainly not normal, and I fear most of them were actually dead. Consideration must be given to the experiments and calculations of the authors as carried out on models which exhibit *some* of the properties of muscles, but they have to be supplemented by *in vivo* experiments.

Hevesy & Rebbe (1940), and later Hevesy, Hahn & Rebbe (1941), studied the exchange of ^{32}P in the frog's gastrocnemius after injection of a radioactive preparation into the lymph sac. The radiophosphate is distributed in less than $\frac{1}{2}$ hr. throughout the extracellular space and penetrates slowly into the muscle fibres where it is built into the organic P compounds. According to the determinations made by the authors the inorganic P of the muscle cells is about 10 times more concentrated than the plasma phosphate,† but the authors themselves are sceptical regarding the result and emphasize the possibility that very labile organic P compounds are really present and simulate inorganic phosphate by being broken down

* I find that Fenn & Cobb (1934) made experiments very similar to those of the second series of B. and C., viz. soaking of sartorii in isotonic Ringer with increasing K content at low temperature. They state expressly that muscles lose their excitability at a K concentration of about 12 mM. in the bathing fluid, and their experimental results are very much like those of B. and C.

† In the paper the ratio is given as 100 times, but this is a misprint.

during the process of isolation. It appears much more probable that the inorganic phosphate concentration within the muscle fibre is in equilibrium with that of the extracellular fluid.

The acid-soluble organic phosphates are renewed at a fairly rapid rate as shown by comparisons between the activity of the inorganic P in the muscle, the creatine-phosphoric acid and the residual acid-soluble P, while the renewal of the phosphatides and the protein P is many times slower. Almost all the creatine-phosphoric acid gets *renewed* in the course of 10 hr., but it takes 4 days for 8% of the total acid-soluble phosphate to be *replaced* by active plasma P, from which it may be concluded that the permeation of phosphate through the surface is a slow process compared with the renewal inside the fibre. We have therefore in the frog muscle a chemical mechanism similar to the one found in mammalian erythrocytes by which the organic P compounds are steadily being broken down and rebuilt and a high concentration of P inside the fibres thereby maintained. If the concentration of inorganic P in the fibre water should be definitely higher than outside, an active transport mechanism must, in addition, be assumed.

The figures available in the paper by Hevesy & Rebbe do not allow any calculation of the permeation constant of the fibre membrane for phosphate, but by combining a number of data from the paper by Hevesy *et al.* (1941) such a calculation can be made as follows for a single experiment comprising two periods of 1 and 4 hr. duration.

Active phosphate of negligible weight was injected into the lymph sac of a frog; 1 hr. later one leg was removed and determinations made on the blood plasma and the gastrocnemius muscle, and after a total of 4 hr. determinations were made on the other leg. The results are given in table 15.

TABLE 15. ACTIVITY OF PLASMA AND GASTROCNEMIUS OF THE FROG 1 HR. AND 4 HR. RESPECTIVELY AFTER THE START OF THE EXPERIMENT. TEMP. 22° C

time, hr.	fraction	fresh weight in mg.	P content in mg.	activity per mg. fresh weight
1	plasma	98.7	0.00344	116*
	gastrocnemius	861.8	1.355	32.7
4	plasma	1267.5	0.044	100
	gastrocnemius	812.4	1.300	93.5

* Taking the 4 hr. value to be 100.

From the weights given a P content in the plasma water of $C_o = 3.5$ mg. % is calculated, and similarly in the fresh gastrocnemius 157 mg. %. Of this quantity two-thirds can be taken as so readily exchangeable that the rate of permeation is the limiting factor for the uptake (inorganic + creatine P, table 11, p. 179), and this is dissolved in the fibre water making up two-thirds of the fresh weight so that we have $C_i = 157$ mg. %. From the figures given combined with the curve (figure 2, p. 156), illustrating the variation in activity of the plasma during the first 4 hr. after

an injection of ^{32}P into the lymph sac, it is estimated that the mean plasma activity during the first hour has been $a_o = 67$, and during the 4 hr. $a_o = 110$.

The activities found in the muscle after 1 and 4 hr. respectively are made up by the extracellular activities and the fibre water activities, which latter are calculated as $a_i = (a_m - 0.13a_o)/0.67$, and work out for 1 hr. $a_i = 26.3$ and for 4 hr. $a_i = 120$. Thus $C_o(a_i/a_o)$ for 1 hr. = 1.4 and for 4 hr. = 3.8. Log q works out as 0.0039 and 0.0106 respectively, and the permeation constants, taking the fibres as cylinders of infinite length and 0.01 cm. diameter, become respectively $P = 2.1$ and 1.5×10^{-5} .

Professor Hevesy informs me that determinations made on working muscles of the rat have revealed no change in the phosphate permeability, a result which is of special interest in view of the fact that the cation permeability of working muscles is definitely increased as discussed below.

(d) *The cation permeability of resting muscles.* Fenn & Cobb (1934) had shown that the K content of muscles depends to some extent on the K concentration of the bathing fluid, and it was shown by Heppel (1939a), in experiments on rats kept for a period of about 45 days on a diet poor in K, that this resulted in a fall in serum K from about 7 to 2.6 mM., and a fall in muscle K (per kg. fresh tissue) from 110 to 64 mM., while the Na increased from about 18 to 54 mM. with no change in the total water content, an increase of about 1 mM. in Cl and a similar fall in P. No further reduction in muscle K could be obtained without seriously affecting the rats. Calculated for the fibre water there is a decrease in K from about 160 to 100 mM. and an increase in Na from a small and doubtful figure to about 50 mM., with a slight swelling of the fibres, compensated by a reduction of the extracellular space. There can be no doubt about the fibre permeability for both ions.

In experiments on isolated frog sartorii soaked for varying periods of time in 50–100 volumes of K-free Ringer, H. B. Steinbach (1940) found that K would gradually leave the muscles and become replaced by Na. His results are calculated for the final net weight of the tissue, and nothing is said about the change in this. The temperature is not mentioned, but it is stated that 'muscles were routinely tested for irritability by single condenser shocks' and 'no particular relationship was observed between loss of potassium and loss of irritability'.

According to his figure 1 he succeeded in reducing the K to about 20 mM./kg. while the Na rose to an average of 80 mM. Down to a content of 30 mM. K/kg. there was a simple exchange with Na, and it must be assumed that the muscles remained excitable. The exchange was found to be a fairly slow process as shown by table 16 compiled from Steinbach's table 2 and figure 1.

It is impossible to doubt the Na permeability, but it can be argued that it might be brought about by the K-free Ringer. The reversibility of the changes observed by Steinbach will be discussed below.

A small number of experiments are available in which radioactive isotopes were utilized to determine permeability. As usual these have to be recalculated to give absolute figures for the constant P and the recalculation involves more or less uncertainty.

Heppel (1939c) determined Na permeability on his K-deprived rats which had been kept on the diet long enough to attain a steady state. He injected a small amount of active NaCl intraperitoneally, killed the rat after a suitable period of time, took samples of serum and muscle (freed from fat), and determined both Na and activity on the ashed samples.

TABLE 16. AVERAGE K AND Na CONTENTS OF FROG SARTORI
SOAKED IN K-FREE RINGER

time hr.	number of analyses	K mM./kg.	Na mM./kg.
0	—	(84.6)	(23.9)*
2	4	71.0	28
5	2	66.5	—
17	10	34.5	65
18	4	33.5	—
19	7	30.0	—
24	4	27.3	70
27	2	28.7	—
?	5	20	82†

* From Boyle & Conway.

† From figure 1.

His results are given per kg. fresh tissue and can be recalculated to serum water and fibre water respectively with very little uncertainty. As a matter of fact, the only significant source of error is the uncertainty regarding the mean serum activity for the experimental period. It is necessary to use the final activity determined, but since the exchange is fairly slow the error cannot be serious. It may make the permeability slightly too high.

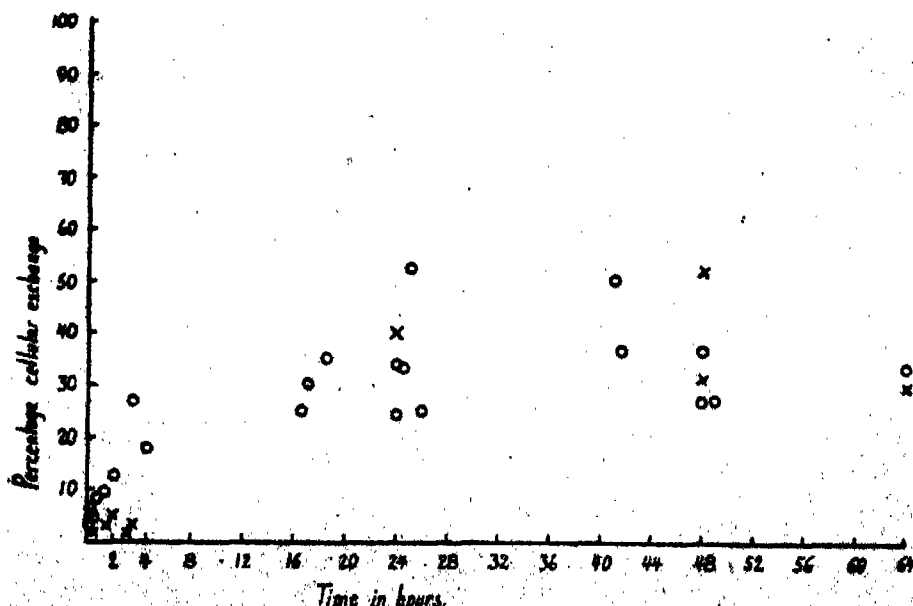
Determinations on six rats lasting from 10 min. to 1 hr. gave the following values for $(\log q)/t$: 1.6, 0.61, 0.79, 0.81, 1.07, 0.83. Excluding the first we find $P = 0.8 \times 0.576 \times 10^{-3} = 4.6 \times 10^{-3}$, a remarkably high figure.

Hevesy & Hahn (1941) studied the exchange of ^{42}K between plasma and tissues in the rabbit and made special determinations of the rate of exchange in muscles. They concluded, on very convincing evidence, that only a certain fraction of the cellular K is able to exchange with the extracellular within the period of 48–64 hr. available for experimentation with the ^{42}K preparations at their disposal. In one series of experiments they determined the rate at which ^{42}K would disappear from the circulation into the tissues allowing for the loss with the excreta. I reproduce their table 5 (table 17).

It is evident that after 24 hr. a steady state is reached with no further cellular exchange, although only 14.8/43 or roughly one-third of the total cellular K is involved. The results obtained vary between about 25 and 53 % exchangeable. In another series they determined the exchange directly on gastrocnemius muscles with substantially the same result. Their determinations on the whole animal are summarized in figure 5 from their paper.

TABLE 17. RATE OF DISAPPEARANCE OF ^{42}K AFTER SUBCUTANEOUS INJECTION FROM THE PLASMA OF A RABBIT WEIGHING 2.4 KG. (RABBIT E)

time hr.	1 g. plasma	% ^{42}K injected present in			ratio of ^{42}K content of the tissue cells and the extracellular fluid
		total extracellular fluid	total extracellular fluid + excreta	tissue cells	
1.8	0.0159	10.4	11.7	88.3	8.5
17.3	0.0106	6.9	12.9	87.1	12.6
24	0.0091	5.9	13.8	86.2	14.6
41.5	0.0080	5.2	17.6	82.4	15.8
49	0.0105	6.8	21.2	78.8	11.6
64	0.0079	5.1	24.5	75.5	14.8



Percentage of the potassium content of the muscle cells which got into exchange equilibrium with the potassium present in the extracellular fluid. \circ represent the values obtained by the "disappearance" method; while \times indicate the figures calculated from the activity values obtained from plasma and muscle tissue.

FIGURE 5

The general significance of these results will be discussed below. At the present stage they are utilized only in the calculation of permeabilities from experiments of short duration in which C_i cannot be taken as the total concentration of K in the fibre water, but at most as one-half of this.

Experiments on rabbits *in vivo* are given with determinations of relative activities of 1 g. muscle and 1 g. plasma after 1–210 min. The very brief experiments are

unsuitable, because the concentrations in the extracellular spaces cannot be considered uniform, and I have selected from their table 8 only experiments on rabbits A, C, H and F lasting 2, 0.68, 3.5, 3.5 and 24 hr. respectively. I have assumed for the plasma water a K concentration $C_o = 5$ mM. K, for the fibre water

$$C_i = 171 \times 0.3 = 51 \text{ mM.}$$

The plasma activity being given as 100 I have adopted $a_i = 106$ for the plasma water, and I have divided the activities given for the muscle cells with 0.72 to obtain the cell water activity a_i .

As before, the plasma activity is the final value and should have been the mean during the experimental period. It is therefore certainly too low, and P is probably overestimated somewhat more than in the ^{24}Na experiments of Heppel. The results are given in table 18, from which it is seen that $P = 230 \times 0.576 \times 10^{-6} = 1.3 \times 10^{-4}$, which is 35 times lower than that found for Na in Heppel's K-deprived rats.

TABLE 18

rabbit	time hr.	$C_o(a_i/a_o)$	$(\log q)/t$
C	0.68	2.55	0.0330
A	2.0	7.15	0.0330
C	3.5	4.9	0.0125
H	3.5	5.75	0.0148
F	24	32	complete exchange

Hahn & Hevesy (1941*b*) published three similar experiments on gastrocnemius muscles of resting rats lasting 48–51 min. In this case the water contents and K concentrations utilized for the calculation are taken from analyses by Fenn & Cobb and come out as $C_o = 4.15$ mM., $C_i = 175/3 = 59$ mM.

The muscle water is 760 g./kg., of which 15.2% = 115 g./kg. water is extracellular. The results come out as $(\log q)/t = 0.10, 0.12, 0.13$ and $P = 7 \times 10^{-4}$.

(e) *The change in permeability during muscular work.* Although a considerable amount of conflicting evidence is found in the literature up to about 10 years ago, the later studies show unmistakably that in contracting muscles the ion permeability is increased, K lost from the fibres and Na gained.

Fenn and his associates (Fenn 1936; Fenn & Cobb 1936; Fenn, Cobb, Manery & Bloor 1937) studied the process on frogs, rats and cats. Their experiments lasted long enough to show that the changes in K and Na were independent of the changes in water content and lactic acid which reach a maximum in a few minutes, but the changes observed were so relatively small that it was found possible to assume that they affected only an 'outer layer' of the fibre and had nothing to do with the permeability of the fibre membrane proper.

Wood, Collins & Moe (1939), working on a gastrocnemius in a heart-lung-muscle preparation of the dog, were able to show that the K loss depends solely on the contraction process and begins immediately when this sets in.

The experiments of Tipton (1938), in which a cat muscle was stimulated indirectly at the rate of 11 shocks/sec., showed that the loss of K and the uptake of an equivalent amount of Na could go on for 3 hr. and involve 30% of the total, and Heppel (1939*b*) showed that in work experiments on his K-deprived rats the K content could be reduced even to 36% of the value for normal rats at rest.

The increased permeability brought about by work was directly demonstrated and measured by Hahn & Hevesy (1941*b*) in experiments in which rats were kept swimming vigorously during the whole period after injecting the radioactive K.

TABLE 19. UPTAKE OF ^{42}K BY THE GASTROCNEMIUS OF RESTING AND SWIMMING RATS

rat	killed after min.	% activity injected present in		ratio of the ^{42}K content of 1 g. muscle and 1 g. plasma	% cellular K exchange
		1 g. plasma	1 g. muscle		
resting (I)	48	0.080	0.102	1.27	6.7
resting (II)	49	0.102	0.162	1.59	7.4
resting (III)	51	0.072	0.122	1.69	7.9
swimming (IV)	47	0.060	0.409	6.82	27.1
swimming (V)	48½	0.088	0.368	4.17	18.5
swimming (VI)	51	0.060	0.492	8.20	39.8

TABLE 20. UPTAKE OF ^{42}K BY THE GASTROCNEMIUS OF RESTING AND SWIMMING RATS

rat	% activity injected present in		ratio of the ^{42}K content of 1 g. muscle and 1 g. plasma	% cellular K exchange
	1 g. plasma	1 g. muscle		
resting (VII)	0.054	0.54	10.0	36
resting (VIII)	0.063	0.63	10.0	37
resting (IX)	0.065	0.63	9.7	35
swimming for 4.9 hr. (X)	0.066	0.61	9.2	37
swimming for 5.7 hr. (XI)	0.055	0.55	10.0	41
swimming for 6.6 hr. (XII)	0.054	0.50	9.3	44

The figures for the resting rats were recalculated above to give the fibre permeability constant $P = 7 \times 10^{-4}$. A corresponding calculation for the swimming rats will give $P = 23 \times 10^{-4}$. Even this is only half the Na permeability calculated from Heppel's figures. In a second series of experiments six rats had ^{42}K injected simultaneously. Three of these were kept practically at rest during 24 hr., the three others were forced to swim in 10–13 30 min. periods distributed regularly over the 24 hr. This should increase the rate of exchange threefold according to the first series, but in the 24 hr. period it made very little difference (see table 20), thus showing that a very large fraction, say 60%, of the fibre K is either non-exchangeable or exchangeable at a rate many times slower than the rest. This point will be discussed below, p. 190.

(f) *The active ion transport in muscles.* There cannot be any rational doubt that when changes in ionic composition are brought about in the muscles of the normal organism either by work, by exposure to a reduced K concentration in the extracellular fluid (Heppel), or in any other way, they are completely reversible, and this was directly shown to be the case by analysis in the experiments of Fenn & Cobb on rat muscles, of Fenn *et al.* and of Tipton on cat muscles, and finally in those of Steinbach on frog muscles *in vitro*. The recovery process means an uptake of K and an elimination of Na, both transports being directed against the concentration gradient. The experiments and calculations of Boyle & Conway make it extremely probable that in the case of the muscles studied it is not necessary to assume an active transport of K. An active transport of Na directed outwards is sufficient, and the analyses show that it will in many cases maintain the Na content of the fibres at a very low level. If such a transport mechanism is always at work and removes Na as fast as it can no special regulating mechanism would seem necessary.

To my mind it appears deeply significant that the frog muscle fibres irreversibly damaged in Boyle & Conway's experiments *became* at the same time impermeable to Na. To reconcile this with any pore permeability conception is certainly difficult, but it may be assumed, in accordance with Lundegårdh's conception, that the special micellae responsible for the Na exchange are in some way prevented from turning round when the excitability is abolished.

(7) *The heart muscle*

Incomplete analyses of the ionic composition of the frog heart and comparisons with the serum are given by Krogh, Lindberg & Schmidt-Nielsen (1944). Frogs (*R. esculenta*) with an average weight of 72 g. had an average heart weight of 180 mg., and toads (*Bufo vulgaris*) of 85 g., 270 mg. The average dry substance was 14.7% and the ash 1.0%. Chloride was determined on four hearts, of which two had been working for about 10 min. with ordinary Ringer, two with a corresponding nitrate solution. The extracellular volume and chloride was determined on each, and the intracellular Cl was calculated as 12 and 18 mM. in the two nitrate-treated hearts and 37 and 48 mM. in the Ringer-treated. The conclusion that the muscle cells are Cl permeable is at least very probable. The cation concentration (including Na + K + most of the Ca, but no Mg) was invariably higher than in the serum, viz. 150–170 mM. for the water of the whole heart, against 130 mM. The amount of extracellular fluid is variable and difficult to determine. From 12 to 40% of the weight was found with an average of 28%. The intracellular cation concentration therefore works out as 165–240 mM. Of this 82 ± 5 mM. is K. The Ca content is very low and, if free, would correspond to a concentration of 3–7 mM. Phosphate was determined on the ash in a few cases to about 70 mM.

In the paper quoted it is concluded that part of the cations must be combined in such a way as not to exert any osmotic pressure, but this conclusion is not binding in view of the uncertainties of the analyses, and it is at least as probable

that the surplus of cations is combined with protein exerting practically no osmotic pressure as in the skeletal muscles.

Hahn & Hevesy (1941a) determined the K content and ^{42}K exchange on the hearts of rabbits and rats. In experiments of short duration (rabbits 3.5 hr.) they find a much faster exchange than in the gastrocnemius muscle (12.5% against 3%), as was to be expected, because the heart muscle is working all the time, but the exchange measured after 48 and 64 hr. is about the same and corresponds only to about 30% of the total, just as in skeletal muscles. The experiments on resting and swimming rats show the same total exchange of about 35% after 24 hr.

A similar very incomplete experiment by Krogh *et al.* (1944) made on an isolated frog heart suggested an exchange of about two-thirds of the total K in 6 hr.

The main series of experiments of these authors show that K can be washed out from the heart down to a cellular concentration of one-third normal or less by repeated changes of a K- and Ca-free solution, while an increase of the K content of the bathing fluid from 1.5 to 6 mM. has very little if any influence on the K content of the heart muscle cells. A variable fraction of the small Ca content can also be removed. The loss in K is replaced by Na. In the process the contractions of the heart are gradually reduced and finally abolished. The washing out process was found to be reversible when it had not gone too far, both with regard to K (and Na) and Ca, when the heart was again provided with normal Ringer. The rate of loss or of active absorption of K was not visibly affected by the presence or absence of glucose.

In experiments in which either the K or the Ca concentration was varied and the working of the heart studied, it was found that the responses to changes in K are gradual and take some minutes to develop while those to Ca changes are much more abrupt, and it is concluded that the K and Ca ions responsible for the effects on rhythm and amplitude are located on the cell surfaces, but can be replaced both from outside and from the interior of the cells. The exchange with the interior is fairly rapid in the case of K, but quite slow in the case of Ca. The actual quantities combined at cell surfaces could not be determined and must be very small. It is not at present possible to decide whether the exchange taking place in the frog heart can be explained by assuming only an active elimination of Na or if it is necessary to assume also an active absorption of K.

In a special series of experiments (Krogh & Lindberg 1944) the results of Sakai (1913), showing that a frog heart can work on a Ringer solution in which five-sevenths of the Na is replaced with glucose, were verified, and it was found that a very large fraction of the Na of the cells was given off to the bathing fluid even to the extent of reducing the intracellular Na concentration below the extracellular. With a normal K content in the bathing fluid the concentration changes of this ion in the heart were negligible. Brought back into normal Ringer the heart would give off glucose and take up Na, but we did not succeed in completely restoring the normal balance.

(8) *Ionic exchanges in other tissues and the state of K in cells*

(a) As mentioned above and shown by the circles in figure 5, Hevesy & Hahn found by injections of ^{42}K on rabbits and determinations of the plasma activity after varying periods of time, up to 64 hr., that the exchange with the organs which was fairly rapid at first would come to a practical standstill after about 24 hr., when only some 30–50 % of the cellular K had been exchanged. The direct determinations on skeletal muscles and on the heart confirm the practical non-exchangeability of about 60 % of the fibre K. Similar determinations were made on the liver in which 11–13 % exchange was observed after $3\frac{1}{2}$ hr. and 31–33 % after 48–64 hr., and it is possible, although in my opinion not proved, that even in the red corpuscles a large fraction of the K is practically non-exchangeable.

The problems raised by these facts are very formidable. They cannot be solved at present, but it may be useful to discuss the possibilities—and the impossibilities.

I think the idea of a K salt of an unknown organic acid and with a very low dissociation constant can be ruled out, because it is known now that even in such a salt the exchange of ions becomes complete in a very short time.

A complex K compound seems to be required in which the K cannot be exchanged except by chemical disintegration.

Since in the muscle all the K is apparently required both to make up the osmotic pressure and as electropositive ions to insure the neutral reaction, one has to imagine a complex soluble K compound of small molecular weight and acting as a cation. Such a substance is unknown, and it seems doubtful if it can exist at all.

It is necessary to consider the possibility that the agreement between the analytical results for muscles, quoted above, and the osmotic pressure is fortuitous, that about 60 % of the K is osmotically inactive and is replaced by organic substances of small molecular weight, which have so far escaped detection. The resulting cation deficit is perhaps not so serious, since it means only that a smaller anion activity has to be ascribed to the proteins.

A further possibility which must be considered is the existence of compartments inside cells bounded by K-impermeable membranes. In muscles the contractile substance proper, making up, according to a personal communication from Buchthal, about 60 % of the fibre, might be conceived as enclosed in such a membrane although the difficulties from the point of view of the contraction theories seem almost unsurmountable.

The idea of K-impermeable structures within muscle cells is given some support by Steinbach's study (1937) of the retractor muscle of *Thyone briareus* (a Holothurian). This is stated to represent an intermediate stage between striated and smooth muscle. It is built up of multinucleate fibres containing 6–12 large fibrils running longitudinally in each fibre.

Steinbach finds the cells to occupy 82 % of the whole muscle and the fibrils 53 %. He soaked the muscles for periods of several hours in isotonic mixtures of sea water and sucrose, varying the Cl and K concentrations. Both Cl and K would diffuse freely into the cells and become evenly distributed in the extrafibrillar

space, but the fibrils, which are chloride free and contain almost the whole of the K, would not take part in the exchange. Experiments with very low K concentrations in the bathing fluid show, however, that the fibrils cannot be really impermeable to K.

In the liver cells the nucleus might be conceived as impermeable, but as the volume of the nucleus is too small to account for the large percentage of K which does not exchange it might be easier to assume that certain cell types in the liver possessed the necessary impermeability.

If we are to accept the non-exchangeability also of a large fraction of the K in red corpuscles the idea of impermeable partitions inside the cells must be ruled out, as an internal structure capable of holding back K ions is certainly absent.

(b) *Chloride in the giant axons of the squid* (Steinbach 1941). In the squid single nerve fibres are found of such dimensions that it is possible, as with the giant plant cells, to make analyses on the content of a single cell, and Steinbach has made Cl determinations by means of Wigglesworth's ultra-microtechnique (1938). The Cl concentration of the fresh nerve averages 36 mM., but shows considerable variation. In fibres kept in isotonic sucrose for a couple of hours the Cl content falls to 0 or very low, but rises again in sea water: 'an artificial sea water made with sodium nitrate and containing 86 mM. Cl also removed excess chloride, a fact of some interest, since the chloride content of the solution was higher than that of the nerves at all times.'

In nerves killed by 60 sec. in absolute alcohol the Cl rises in sea water, but takes a long time (more than $1\frac{1}{2}$ hr.) to become equalized with the outside: 'The most plausible interpretation of the results involves the assumption of a steady state involving definite diffusion forces. The chloride balance of cells is thus to be considered as a part of the general series of mechanisms responsible for the selective partition of other ions such as potassium and sodium.' 'Once it has been demonstrated that a given cell admits chloride or any other ion during some stage of its existence, without, at the same time, coming to a complete diffusion equilibrium with the external environment, then it is clearly an unnecessary complication to postulate an impermeable membrane surrounding the cell during any period. Active forces must be at work during any permeable "phase" in the history of the cell and when the necessity for these forces is recognized there is nothing to be gained by adding other unknown factors, until specific evidence is brought forth for their existence.'

I am in complete accord with the opinions so well expressed by Steinbach.

(c) Results which are of considerable interest from the point of view of K exchange are reported by Hoagland & Rubin (1936). Hoagland had found that mechanical stimulation of frog skin by an air jet, interrupted 140 times per second, produced inhibition of the response to tactile stimuli (action current in nerves) which took about a minute to recover.

This inhibition could not be due to any process in the nerve, and it was thought that it might be an ion effect on the tactile end-organ. Consequently the inner side

of a piece of skin 1×2 cm. was washed with Ringer during stimulation for 3 min. and the Ringer afterwards analysed. No change was found for Ca, PO_4 or Cl, but a distinct increase for K amounting on an average to 67% corresponding to a 9% increase in the K of 10 ml. Ringer, while controls gave only 6%. K is known to inhibit tactile endings.

A paper by Rubin (1936) gives the ionic content of frog skin (*R. pipiens*) calculated as mg. % of the wet weight. Unfortunately, the content of dry substance is not given. I have therefore supplemented the analysis by a determination of dry substance of frog skin (*R. temporaria*) kindly carried out for me by Mr Flodmark, who found that 3.14 cm^2 skin weighed 127.5 mg. fresh and 22.7 mg. dry, corresponding to a water content of 82.4%. Utilizing these figures Rubin's results can be recalculated into the following contents of 1 kg. skin water in mM., viz. 41 K, 48 Na, 92 Ca, 59 Cl and 132 P.

The piece of skin in Hoagland's and Rubin's experiment should contain 107%, of which 61 or 57% is liberated during the 3 min. stimulation. This K must be given off from the epithelial cells surrounding the nerve endings and must in the living animals ultimately become restored to these by an active process.

I have no doubt that a systematic search in the literature would reveal several further examples of active ion transport in connexion with the functions of normal grown cells, but those now given will have to suffice, the more so as during cell development and growth active ion transport must be of almost universal occurrence.

SUMMARY AND CONCLUSIONS

The power of active transport of ions is of common occurrence both in the vegetable and in the animal kingdom and is possibly a general characteristic of the protoplasmic surface membrane.

A distinction is made between transport *through* cellular membranes into and out of whole organisms and transport into and out of single cells. Transports through the protoplasmic lining of the huge cells of certain algae form a connecting link and the distinction is perhaps purely formal.

It is contended that where large differences in concentration of single ions are maintained between organisms and the surrounding water or between cells and the surrounding extracellular fluid, one has to do not with a true equilibrium, but with a *steady state* maintained against a passive diffusion and requiring the expenditure of energy.

Certain membranes are discussed which show—so far as at present known—only passive permeability. Of these the capillary endothelium and the glomerular syncytium in the kidneys are freely permeable to ions and show varying resistances to colloids, while the blood vessels of the central nervous system in vertebrates are very slowly permeated by inorganic ions, and at the same time freely permeable to water and to lipid-soluble substances. An active ion transport must take place somewhere in the central nervous system as evidenced by the differences between the cerebrospinal fluid and the blood, but has not so far been definitely located.

In the roots of higher plants a transport of ions from the soil solution into the sap rising from the roots through the stems takes place regularly. In many cases this transport is active, as concentrations in the sap are definitely in excess of those in the outside fluid, both as regards single ions and the sum of ions.

In fresh-water animals belonging to all larger systematic groups mechanisms have been demonstrated for the absorption, from solutions which are usually extremely dilute, of certain ions to the exclusion of others. An independent mechanism is described for the gills of fishes absorbing Cl^- and Br^- , but excluding NO_3^- , I^- and CNS^- , while another mechanism absorbs Na^+ , but excludes K^+ . Quite similar mechanisms are found in Amphibia and in many invertebrates, but in a recent invader of fresh water (*Eriocheir*) the mechanisms both for anions and for cations are much less selective.

In aquatic arthropods the cation-absorbing mechanism takes up Ag^+ by which ion it seems to be blocked.

In the kidney tubules specific mechanisms for absorption against a gradient of a number of ions appear to be located and to be mutually independent.

In the mammalian ileum a mechanism for the uptake of univalent ions evidently exists (Visscher *et al.*), but its mode of operation and specificity is still controversial. The views put forward are discussed and the desirability of further experimentation emphasized.

The passive ion permeability of all the membranes so far dealt with has not been quantitatively studied. It seems to be low in most cases, but high in plant roots and fairly high in the gills of *Eriocheir*.

In the study of single cells one must distinguish between plant cells (at least the overwhelming majority of them) and almost all animal cells.

The former are enclosed in a rigid cellulose wall capable of standing a high hydrostatic pressure, while the latter are naked and able to sustain only a negligible pressure. In the former the total osmotic concentration may therefore be, and usually is, much higher than that of the extracellular fluid, giving rise to the characteristic turgor. It is characteristic also of plant cells that they grow to a comparatively large size and enclose a large vacuole containing sap with a very low concentration of protein or other high molecular substances. In the latter there is no vacuole; the ions and other low molecular solutes are dissolved in the 'free' water of the cells which seems in most cases to be practically the same as the total water. As a necessary consequence of the surface membrane being freely permeable to water the total osmotic concentration must always be the same inside and outside.

The comparison of a number of giant cells from algae, the sap of which can be isolated and analysed, shows that almost any ion can be either concentrated in the sap or kept out of it, but the most clear-cut and quantitative results were obtained by the study of Characeae (Collander) from brackish and fresh water in which the sap is an almost pure salt solution usually some 200 mM. more concentrated than

the fluid in which the algae are growing. During the growth of such cells from a microscopic size to a volume of $50\mu\text{l}$. a number of ions are taken up and concentrated, some of them more than a thousand times; but even when growth has ceased the uptake continues, making good the loss by diffusion to the outside and sometimes increasing the turgor to the bursting point.

In spite of the simultaneous processes of active ion uptake the passive permeability for any ion can be determined, provided an isotope is available which can be determined by its radioactivity or in any other way (Hevesy). The necessary condition is that a steady state with regard to the ion in question is reached before the experiment and maintained throughout it. Experiments of this type with radioactive isotopes of Na and K on *Tolypellopsis* and *Nitella* are described and the permeabilities expressed in absolute units, cm./hr., according to the formula

$$P = \frac{\text{volume}}{\text{surface} \times \text{time}} \log_n \frac{\text{concentration in sap } (C_s)}{C_s - C_{\text{outside}} (\text{activity } s / \text{activity } o)}$$

The resulting permeabilities are of a low order (between 18 and 0.1×10^{-5} cm./hr.), lower for the fresh-water than for the brackish-water form, and lower for Na than for K.

At least the large majority of plant cells accumulate and concentrate ions in the sap during growth. It is generally assumed that the accumulation stops when growth ceases. This would involve an absolute impermeability to the ions accumulated, and it is much more likely that a steady state is attained in which the loss by diffusion is made good by continued accumulation. This point is open to study by means of isotopes, but the adsorption of ions to the cell surface presents difficulties which will have to be overcome.

The roots of plants are specially adapted for active absorption of ions which are partly carried on together with water as the sap rising through the stems, partly stored in the growing roots.

Lundegårdh has brought forward conclusive evidence in the cases studied by him to show that anions are actively transported while the cations are electrostatically attracted. The energy necessary to concentrate anions varies; it is smaller for monovalent than for divalent ions and NO_3^- appears to be most easily absorbed, but this may be due to the fact that its concentration in the sap is kept relatively low, because a large fraction is immediately utilized in organic syntheses. Also the cations are as a rule taken up independently of the outside concentrations, and K in most cases preferentially, but so far as has been ascertained no ions are definitely left out. The ion absorption is independent of the simultaneous uptake of water, which is largely osmotic, but may be to some extent active.

The permeability of the roots for ions appears to be very high, and the expenditure of energy necessary for the uptake is considerable.

Lundegårdh suggests on the basis of his studies, also of electric potentials in the roots, a special dynamic structure of the surface membrane which is compared with a Langmuir monomolecular film composed of long lipid molecules. The root surface

film must be mosaic and present both anionic and cationic hydrophile atomic groups kept separate in the membrane, but each of them capable of exchanging ions attached to them with the interior when they turn round in the membrane. If the Lundegårdh membrane conception is to be extended to cover also animal membranes and cell surfaces it becomes necessary to assume molecules (or micellae) with highly specialized ionic affinities.

The types of animal cells discussed comprise a number of Protozoa, certain animal eggs, mammalian red cells, the chorion membrane from hens' eggs, certain vertebrate striated muscles, the heart muscle and some other organs, the giant axons of the squid and the skin of frogs. In all these cases evidence is presented indicating an active ion transport.

In the cases of erythrocytes and muscles for which it is possible to determine the relation volume/surface with a fair degree of accuracy, and for which also the concentrations of certain ions (HPO_4 , K and Na) both in the extracellular fluid and in the cell water can be taken as approximately constant and known, the permeability determinations by means of isotopes, found in literature, are recalculated and expressed in absolute units.

In the case of phosphate the investigations of Hevesy and his collaborators show a definite but slight permeation both in erythrocytes and muscles. For frog muscles a recalculation gives $P = 2.1 - 1.5 \times 10^{-5}$ cm./hr. It appears probable that the inorganic phosphate concentrations inside and outside the cells are in equilibrium, but inside a number of organic indiffusible phosphates (creatine phosphate, hexose phosphate, etc.) have an aggregate concentration many times higher than the inorganic. These compounds are unstable and are constantly being broken down and rebuilt at rates which can be measured by the incorporation of ^{32}P and which are for some of the compounds more rapid than the rate of phosphate permeation. A steady state is kept up by the expenditure of chemical energy, but the transport itself is probably a simple diffusion process.

With regard to the cations there is no evidence that either K or Na exist inside the cells otherwise than as free ions, and the analyses show them to be necessary to make up the osmotic pressure to the same value as that of the extracellular solution.

The red corpuscles studied are permeable to both Na and K. Their ionic composition varies with their age and the K content is generally higher in young corpuscles. For K very low permeabilities are deduced from the published determinations, viz. rabbit, 2.5×10^{-7} (*in vivo*, too low, Dean *et al.*); rabbit, $6-10 \times 10^{-7}$ (*in vivo*, Hevesy & Hahn); dog, 6×10^{-8} (*in vivo*, Hahn & Hevesy). For Na the figures are definitely higher, viz. rabbit, about 10^{-4} (*in vivo*, Hahn & Hevesy); dog, 1.4×10^{-5} (Cohn & Cohn); dog, 2×10^{-5} (Hahn & Hevesy).

The results, which are incompatible with a pore permeability conception, are definitely supported by experiments reported by other writers.

K is lost from human or rabbit erythrocytes at low temperature and at body temperature when glucose is absent, but can be recovered even *in vitro* when glycolysis is going on. Also corpuscles which have been damaged *in vivo* by

distilled water or lead poisoning and have lost K can recover and reabsorb K. When K is lost Na will enter in its place and become eliminated during recovery. No definite evidence is available to show whether both Na and K or only one of these ions is actively transported, but a K transport appears most likely.

Chorion membranes, from hens' eggs incubated 9 days, lose K to a K-free solution, but reabsorb it from low concentrations in the presence of glucose or lactic acid. Like erythrocytes the membranes will lose K at low temperature irrespective of the outside concentration and the presence of sugar.

The fibres of skeletal muscles contain a large surplus of cations over crystalloid anions, but the sum of both, as revealed by analyses, corresponds closely to the⁴ osmotic concentration of the outside fluid. The anion deficit is taken to be made up by colloid proteins.

Boyle & Conway succeeded in quantitatively describing as a simple physico-chemical system the behaviour of sartorius fibres of the frog in solutions with increasing K concentrations, from which the fibres would take up K against the gradient, on the assumption, verified in their experiments, that the fibres were impermeable to Na and larger ions, but permeable to K and chloride. Their equations would only hold, however, at K concentrations above 12 mM., at which the fibres are shown irreversibly to lose their excitability.

Since the permeability to Na of muscle fibres *in vivo* has been abundantly demonstrated, it appears that the assumption of an active elimination of Na with simple diffusion of K can account for the exchanges observed.

Lack of K in the extracellular fluid reduces gradually the K content of muscles which becomes partially replaced by Na, as shown by Heppel for rats living for several weeks on a diet low in K, and by Steinbach on frog muscles *in vitro*.

Heppel demonstrated by means of ²⁴Na on his K-deprived rats a Na permeability of the muscle fibres which is recalculated to $P = 4.6 \times 10^{-3}$ cm./hr., while Hahn & Hevesy by means of ⁴²K found for normal rat muscles a K permeability of 7×10^{-4} and for rabbit muscles 1.3×10^{-4} .

It is a very significant fact that the exchange of K in muscles does not tend towards completion, but comes apparently to a standstill when on an average about 40 % (30–50) of the total is exchanged.

During muscular work K is constantly leaving the muscle fibres, becoming replaced by Na, and in the experiments of Tipton on a cat muscle this went on for 3 hr. and involved 30 % of the total. This is associated with and is perhaps due to an increased permeability, which was demonstrated by Hahn & Hevesy on swimming rats, and from their figures a fibre permeability of $P = 23 \times 10^{-4}$ can be deduced. On the other hand, the final exchange of about 40 % was not affected by forcing the rats to swim for 6 hr. out of 24.

The changes brought about by work in a normal organism are of course completely reversible and have been shown to be so in several published experiments.

The restricted exchange of K is found also in the heart and liver and may even exist in blood corpuscles, although the evidence for this is scarcely conclusive. To

account for this restricted exchange it seems necessary to assume either the existence of compartments within the cells having a much lower permeability for K or the existence of complex compounds the K of which cannot exchange without chemical breakdown.

The muscle-fibre water of the frog heart contains a total of about 200 mM. cations, of which 82 mM. is K and only 3–7 mM. Ca, of which, moreover, a considerable fraction is probably not in solution at all. The Cl concentration is about 40 mM. and the total phosphate (organic and inorganic) about 70 mM. It is to be assumed that the anion deficit is, as in skeletal muscles, made up by proteins.

A large part of the Cl can diffuse out when nitrate is substituted for Cl in the fluid on which the heart has to work.

K can be reduced to one-third or less and replaced by Na when the heart is supplied with K- and Ca-free Ringer and this slowly brings the heart to a standstill. A variable fraction of the small Ca content is also removed. The washing out process is completely reversible with the recovery of contractility when it has not gone too far. The rate of loss or recovery of K was not found to be affected by the presence or absence of glucose.

The K/Ca balance responsible for the contractility refers to ions 'adsorbed' in the fibre surface, and these can be replaced both from outside and from the inside of the fibres. In the case of K the replacement is retarded by the ion movement through the fibre surface, but in the case of Ca the replacement from outside is practically instantaneous while that from the inside is quite slow.

Special experiments verified Sakai's observation that the heart can work on a solution in which 75 % of the Na is replaced by glucose, and in this case a considerable and reversible exchange of Na with glucose takes place inside the fibres.

In the giant axons of squids Steinbach was able to demonstrate a transport of Cl out of the fibres against the gradient, and in the skin of the frog it was shown by Hoagland & Rubin that weak mechanical stimulation would liberate large amounts of K from epithelial cells which must, as in muscles, become restored upon recovery.

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The transmission of beet mosaic and beet yellows viruses by aphides; a comparative study of a non-persistent and a persistent virus having host plants and vectors in common

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The vectors of beet mosaic virus are optimally infective when they have fed for only a few minutes on the infected plants after a period of fasting. After infection feeding, infectivity is very rapidly lost when the vectors feed on healthy plants, but while it remains a single vector can infect several plants. Infectivity is lost much more slowly when the vectors fast after infection feeding.

In this behaviour beet mosaic virus resembles Hy 3, potato Y, cucumber 1, and other aphid-transmitted viruses which have been called the non-persistent group. It resembles these viruses also in its physical properties.

In some secondary characters beet mosaic differs from the other non-persistent viruses more than they differ from each other. It is retained longer by the fasting vectors, and infectivity of the vectors may increase considerably with increasing infection feeding time, in the absence of preliminary fasting, though it rarely reaches the optimal level.

With beet yellows virus infectivity of the vectors is not affected by preliminary fasting, but always increases with increasing feeding time on both infected and healthy plants. Infectivity increases with increasing feeding time on the healthy plants whatever the infection feeding time, and therefore there is always a delay in the production of optimum infectivity by the aphides after cessation of infection feeding. Infectivity is more rapidly lost from the fasting than from the feeding vectors.

The properties indicate that beet yellows belongs to the persistent group of viruses, although its persistence in the fasting vectors is only about the same as that of beet mosaic, which is a non-persistent virus. The main basis of distinction between the two types seems not to be the time for which they are retained by vectors, but the effect of preliminary fasting.

Beet yellows and beet mosaic viruses have the same vector and host plant and therefore the differences in their behaviour are properties of the viruses themselves, and are not induced by the conditions in which they are transmitted.

In previous papers (Watson & Roberts 1939; Watson 1940), two kinds of insect-transmitted viruses with different vector relationships have been distinguished. These were called 'persistent' and 'non-persistent' viruses, because the most obvious difference between them was the length of time for which the vectors remain infective after ceasing to feed on the infected plants.

Sugar-beet yellows is an example of a persistent virus (Watson 1940). Infectivity of the vector of this virus increases with increasing feeding time on infected plants, and the vectors do not become optimally infective until some time after they have started to feed on the healthy plants. This delay in development of optimum infectivity is independent of the time of feeding on the infected plant. The vectors remain infective for about 72 hr. or less, depending to some extent on the time of feeding on the infected plants. Beet yellows virus is not transmissible mechanically by sap inoculation, but some of its properties have been determined by serological methods (Kleczkowski & Watson 1944); it is inactivated at about 50° C, and remains active in extracted sap at room temperature for about 6 days.

The viruses classed as non-persistent were *Hyoscyamus* virus 3, potato virus Y, cucumber virus 1, and tobacco etch virus, all of which were readily transmissible by mechanical inoculation. With these viruses the vectors are most efficient when, after a period of fasting, they are fed for only a few minutes on the infected plants. With longer feeding infectivity decreases. The vectors usually retain infectivity for about 1 hr. when feeding continuously on healthy plants, but longer when they are fasting. These four viruses have similar properties *in vitro*. In extracted sap they are inactivated by keeping for a few days at room temperature, or by 10 min. heating at about 58° C. They are not serologically related to each other.

These non-persistent viruses and beet yellows virus have a common vector in *Myzus persicae* (Sulz.), and the difference in their vector-virus relationships is believed to arise solely from differences in the properties of the viruses. As they have no common host, however, it is possible that the differences might depend to some extent on the host plants. From its general properties, it seemed likely that beet mosaic virus was non-persistent; the experiments described in this paper were made to test this, and to compare and contrast the behaviour of this virus with that of other non-persistent viruses in different host plants, and with beet yellows virus in the same host plant.

MATERIAL AND METHODS

The beet mosaic virus used in these experiments was obtained from mangold plants grown on Rothamsted farm. The virus is common in sugar beet and mangold crops in Britain, and it also infects spinach, spinach beet, and some chenopodiaceous weeds. It has been described in this country by K. M. Smith (1937) and is probably the same as the beet mosaic described in the U.S.A. by Robbins (1921). In sugar beet the first symptoms are clearing of the veins of the youngest leaves followed by diffuse mottling over the whole plant, often with dark green areas along the veins,

or minute light green circles on a darker background. The virus is transmissible by sap inoculation, and sugar-beet leaves inoculated by rubbing show no local lesions unless killed, decolorized, and stained with iodine. Longevity *in vitro* (3–4 days), and thermal inactivation point (about 58° C), are similar to those of the non-persistent viruses. It is transmitted by *Myzus persicae* (Sulz.), *Aphis fabae* (Scop.) and *Myzus circumflexus* (Theobald).

The meanings of the special terms used in the text are as follows: infection feeding (I.F.)—the act of feeding on the infected plants; test feeding—feeding on the healthy plants after I.F.; preliminary fasting—a period without food before I.F.; post-infection fasting—a period without food between I.F. and test feeding.

In all the experiments *M. persicae* was used as the vector, and sugar beet as the source of infection and test plant. The methods used in culturing and handling the aphides have been described previously (Watson 1938, 1940). All short feeding periods were timed from the moment of penetration of the leaf by the aphid. The time spent in reaching the feeding position was generally recorded, and it was always insufficient to account for the variations in the behaviour of the aphides.

The factorial designs used in some of the experiments, and the methods of carrying out these and the consecutive infection experiments have also been described previously (*loc. cit.*).

The principle of factorial design is that the treatments tested consist of all combinations of a number of factors at varying intensities. The description of each experiment begins with a list of the factors investigated, giving the variants of each. The experiments were mainly concerned with the same factors, but one or more of the factors were held constant in each experiment; these are also given in the descriptions. Each experiment consisted of a number of replications, carried out on different dates, in which each treatment was applied to the same number of plants. Therefore the number of plants used for each combination of treatments is the number of plants used in each replication multiplied by the number of replications; e.g. in experiment 1, 5 plants per treatment \times 6 replications gives 30 plants for each combination of treatments. The figures given in the tables are the numbers of plants infected out of the total number used for each combination of treatments.

The experiments are grouped in four sections according to the general treatment of the aphides.

I. EFFECT OF VARYING PRELIMINARY FASTING AND INFECTION FEEDING TIMES

Experiment 1 (table 1). Beet mosaic virus

Treatments—all combinations of:

- (1) Three preliminary fasting times: 0, 1, 20 hr.
- (2) Four infection feeding times: 2, 15 min., 1, 20 hr.

Constant: test feeding time, 24 hr.

Aphides: 1 per plant.

Plants: 5 per treatment, 6 replications.

TABLE 1. THE EFFECT OF VARYING TIMES OF PRELIMINARY FASTING AND INFECTION FEEDING ON THE TRANSMISSION OF BEET MOSAIC VIRUS BY *M. PERSICAE*

preliminary fasting times	infection feeding time				total
	2 min.	15 min.	1 hr.	20 hr.	
0	2	4	2	6	14 (a)
1 hr.	15	3	3	10	31
20 hr.	15	6	4	12	37
total	32	13	9	28 (b)	82

Standard errors (a) ± 3.07 , (b) ± 2.66 .

The number of plants infected with beet mosaic virus was greatly increased by 1 hr. preliminary fasting, but further increase after an additional 19 hr. was only slight. Aphides which fasted before I.F. gave about 65 % fewer infections after 1 hr. I.F. than after 2 min., but their capacity to cause infection increased again between 1 and 20 hr. I.F. When there was no preliminary fasting the effect of increase of I.F. time was small.

Experiment 2 contrasts the behaviour of beet mosaic and beet yellows viruses in response to varying fasting and feeding conditions. The first part, experiment 2a, shows the effect of varying I.F. time with preliminary fasting constant; in the second part, experiment 2b, both I.F. and preliminary fasting times are varied.

Experiment 2 (table 2). Beet mosaic and beet yellows viruses

(a) Treatments—all combinations of:

(1) Beet mosaic and beet yellows viruses.

(2) Three infection feeding times: 3, 30 min., 24 hr.

Constant: preliminary fasting time, 4 hr.; test feeding time, 24 hr.

Aphides: mosaic 1 per plant, yellows 5 per plant.

Plants: 10 per treatment, 4 replications.

(b) Treatments—all combinations of:

(1) Beet mosaic and beet yellows viruses.

(2) Two preliminary fasting times: 0, 16 hr.

(3) Four infection feeding times: 3 min., 1, 5, 20 hr.

Constant: test feeding time, 24 hr.

Aphides: mosaic 1 per plant, yellows 5 per plant.

Plants: 7 per treatment, 4 replications.

In experiment 2 beet mosaic virus behaved as in experiment 1. The greatest number of plants was infected by aphides which had received preliminary fasting and short I.F.; the number decreased with longer I.F. times up to 1 hr., and then increased again between 1 and 24 hr. I.F. When there had been no preliminary fasting few plants were infected after 2 min. I.F., but the number after 24 hr. I.F. was the same as when the aphides had fasted before I.F.

TABLE 2. THE EFFECT OF PRELIMINARY FASTING AND VARYING INFECTION FEEDING TIMES ON THE INFECTIVITY OF *M. PERSICAE* COMPARED FOR BEET YELLOWS AND BEET MOSAIC VIRUSES

		infection feeding times after 4 hr. preliminary fasting				
		5 min.	30 min.	24 hr.		total
(a)	B.Y.V.	1	3	18		22
	B.M.V.	17	5	8		30

		preliminary fasting times	infection feeding times				
			5 min.	1 hr.	5 hr.	24 hr.	total
(b)	B.Y.V.	none	1 (a)	5	16	25	47 (b)
		16 hr.	1	6	20	24	51
	B.M.V.	none	6	7	11	15	39
		16 hr.	21	6	11	17	55

Standard errors (a) ± 2.96 , (b) ± 5.92 .

With beet yellows virus preliminary fasting had no effect in varying the time at which optimum infectivity occurred. Whether the aphides fasted or not, the number of plants infected after a short I.F. time was negligible. Preliminary fasting did not affect either the rate of increase or the level of infectivity which was eventually reached.

About the same numbers of plants became infected with beet mosaic and beet yellows viruses, but the mosaic virus was transmitted by 1 and the yellows virus by 5 aphides per plant. In these conditions, therefore, *M. persicae* is a more successful vector of beet mosaic than of beet yellows virus, at least when the capacity of the aphides is determined from single and not from consecutive test feedings.

The fact that the transmission of beet mosaic virus was greatly affected by preliminary fasting and that of yellows virus was not, shows that the effect could not have been directly one of 'appetite', that is, increased rate of feeding induced by starvation. Obviously some other factor is involved in the effect of preliminary fasting.

II. CONSECUTIVE INFECTION EXPERIMENTS

Experiments 3 and 4 were made to test the effect of varying fasting and feeding treatments on the capacity of the aphides to transmit the viruses to several healthy plants in succession after a single I.F.

Experiment 3 (table 3). Beet mosaic virus

Treatments—all combinations of:

- (1) Two infection feeding times: 3 min., 20 hr. (trials 1 and 2).
- (2) Two test feeding times on successive plants: 5, 50 min. (trial 1 only).

Constant: preliminary fasting time, 16 hr.

Note. Each aphid fed for the same test feeding time on the first five successive plants and for 16 hr. on the last plant.

TABLE 3. NUMBER AND DISTRIBUTION OF SUCCESSIVE INFECTIONS CAUSED BY *M. PERSICAE* TRANSMITTING BEET MOSAIC VIRUS

N.B. In trial 1, thirty-six aphides were tested for each test feeding time, and in trial 2, made with short test feeding times only, twenty aphides were tested. Aphides which failed to give infection are not included in the table.

Performance at successive 5 min. test feeding periods

successive plants ...	3 min. infection feeding							20 hr. infection feeding						
	1	2	3	4	5	6	frequency*	1	2	3	4	5	6	frequency
trial 1	+	0	0	0	0	0	5	+	0	0	0	0	0	3
	+	+	0	0	0	0	1	+	+	+	+	0	0	1
	+	0	+	0	0	0	1	0	+	0	0	0	0	2
	+	+	0	+	0	0	1	0	0	+	+	0	0	1
	+	+	+	+	0	0	1	0	0	0	0	+	0	1
	+	+	0	0	0	+	1							
	+	+	+	0	0	0	1							
	0	+	+	0	0	0	1							
infections on successive plants	11	6	4	2	0	1	total 24	4	3	2	2	1	0	total 12
total number of aphides infective							12							8
trial 2	+	0	0	0	0		4	+	0	0	0	0		3
	+	+	0	0	0		3	0	+	0	0	0		2
	+	0	+	0	0		1	0	0	+	0	0		1
	+	+	+	0	0		1	0	+	+	0	0		1
								0	0	0	+	0		1
infections on successive plants	9	4	2	0	0		total 15	3	3	2	1	0		total 9
total number of aphides infective							9							8

Performance at successive 50 min. test feeding periods

trial 1	+	0	0	0	0	0	11	+	0	0	0	0	0	9
								0	+	0	0	0	0	1
infections on successive plants	11	0	0	0	0	0	total 11	9	1	0	0	0	0	total 10
total number of aphides infective							11							10

* Columns headed 'frequency' give the number of times each distribution occurred.

There are two aspects of these experiments to be considered. One is the effect of the treatments on the ability of individual aphides to cause consecutive infections, and the other is their effect on the total numbers of plants infected, i.e. the efficiency of the aphides considered as a group. Thus varying the time of test feeding had little effect on the number of aphides which became infective, but the ability of the aphides to cause consecutive infections was greatly affected. When the test feeding time was 50 min. (trial 1), twenty-one aphides caused infection but they infected only one plant each; when the test feeding time was 5 min., twenty aphides caused infection, but half of them infected more than one plant.

Varying the I.F. time also had comparatively little effect on the number of aphides which became infective. Aphides given 20 hr. I.F. infected slightly fewer plants than those given 3 min.; no 1 hr. I.F. treatment was used, so that the aphides were not tested in conditions of minimum vector efficiency. However, when the test feeding time was 5 min., varying the I.F. period did affect the capacity of the aphides to cause consecutive infections. The twenty-one aphides which became infected after 3 min. I.F. in trials 1 and 2 together infected a total of thirty-nine plants, but the sixteen which became infective after 20 hr. I.F. infected only twenty-one plants.

After preliminary fasting and short I.F. time, most of the aphides caused infection during the first 5 min. of feeding if they were going to infect at all. Therefore the vector efficiency of the aphides as a group did not increase with increasing total test feeding time, that is, with the increasing sum of all the times spent on the successive plants. After a long I.F. time, during which the influence of preliminary fasting had been lost, many of the aphides fed on one or more consecutive plants before causing infection, and there were more aphides which had caused infection by the end of the experiment than there were at the beginning. Thus the number of aphides which caused infections increased with increasing total test feeding time.

With Hy 3 virus (Watson 1936) a slight increase in infectivity of the vectors with increasing test feeding time was observed when there had been no preliminary fasting. In later experiments, when preliminary fasting treatments were used, there was no such increase.

Table 4 shows the performance of vectors of Hy 3, potato Y, and severe etch viruses, in consecutive 5 min. test feedings after preliminary fasting and 2 or 3 min. I.F. periods, compared with results for beet mosaic virus obtained in similar fasting and feeding conditions (experiment 3, trial 1).

TABLE 4. NUMBERS OF SUCCESSIVE PLANTS INFECTED BY *M. persicae* IN 5 MIN. FEEDINGS GIVEN AFTER PRELIMINARY FASTING AND SHORT INFECTION FEEDING. COMPARED FOR BEET MOSAIC, *HYOSCYAMUS* 3, POTATO Y AND TOBACCO ETCH VIRUSES

Figures in brackets show the number of aphides used in each experiment, at the rate of 1 per plant.

		successive healthy plants									
		1	2	3	4	5	6	7	8	9	10
beet mosaic virus	(36)	11	6	4	2	0	1	—	—	—	—
<i>Hyoscyamus</i> 3 virus*	(36)	23	12	10	9	8	6	3	1	1	4
potato Y virus*	(24)	13	11	6	6	3	1	2	2	—	—
tobacco etch virus†	(30)	18	8	5	2	3	—	—	—	—	—

* Watson & Roberts (1940).

† Kassanis (1941).

Table 4 shows that *M. persicae* was a less successful vector of beet mosaic than of the other viruses, but otherwise the results were similar. With all the viruses, after preliminary fasting and short I.F. time, most of the vectors infected the first

plants on which they fed and subsequent infections were all caused by these aphides, so that the number of aphides which caused infection did not increase with total test feeding time.

The rate of loss of infectivity from the aphides while feeding seemed to be about the same for all the viruses, but initial infectivity of beet mosaic virus was low and was exhausted on the first five or six plants. With the other viruses infectivity was retained longer, and also its rate of loss became reduced after the first five or six consecutive feedings. At this time individual aphides were weakly infective; they could cause only one, or rarely two, infections among the last four plants on which they were fed, but these infections were produced after longer intervals of feeding than at first, so that the infectivity of the group was reduced less rapidly. Also, more of the last plants, on which the aphides were fed for about 20 hr., became infected than those immediately preceding them, which confirms that the infectivity of the aphides was then being influenced by the test feeding time. In this, the behaviour of aphides after preliminary fasting and short I.F. at the end of a series of consecutive test feedings, resembled that of aphides transmitting beet mosaic virus in short consecutive test feedings after long I.F., and the determining factor seems to be the length of time which has elapsed since the preliminary fasting treatment.

To sum up, it seems that with non-persistent viruses after preliminary fasting and short I.F. periods aphides contain much active virus, but lose it very rapidly. When the influence of fasting before I.F. is nullified by a further period of feeding on either infected or healthy plants, the aphides contain very much less active virus, but it is lost more slowly.

Experiment 4 was made to determine the distribution of infection obtained with beet yellows virus in successive test feedings in order to compare it with that of beet mosaic virus. The time scale over which changes in the performance of the aphides take place with the two viruses is so different that they could not satisfactorily be compared in the same experiment. With beet yellows virus 1 hr. test feeding causes about the same proportional change in the performance of aphides as 5 min. feeding with beet mosaic virus, and this seemed to be the best basis of comparison.

Experiment 4 (table 5). Beet yellows virus

Treatments—constant.

Infection feeding time: 24 hr.

Test feeding time: 1 hr. on five successive plants; 16 hr. on the 6th plant, and 1 hr. on the 7th and 8th.

Aphides: 1 per plant.

Plants: 10 sets of 8 successive plants; 7 replications.

The distribution of infection with beet yellows virus resembled that of beet mosaic virus after long I.F., in that the aphides developed infectivity after varying times from the beginning of the consecutive test feedings; but once the aphides had infected a plant they generally infected several more, so that the total number of

plants infected at each transfer increased for the first 2 or 3 hr. With beet mosaic virus, although there is an increase of infectivity with increasing feeding time, the aphides are only weakly infective, and rarely infect more than one plant in succession, so that the number of plants infected does not increase, although the number of aphides exhibiting infectivity does so.

TABLE 5. NUMBER AND DISTRIBUTION OF SUCCESSIVE INFECTIONS CAUSED BY *M. PERSICAE* IN TRANSMITTING BEET YELLOWS VIRUS

Performance at successive 1 hr. test feeding periods.									
plants ...	1	2	3	4	5	6	(16 hr.)	7	8
	+	0	0	0	0	0		0	0
	+	+	0	0	0	0		0	0
	+	+	0	0	+	0		0	—
	+	+	+	0	0	+		+	0
	+	0	+	0	+	0		0	+
	+	0	0	+	0	+		—	—
	0	+	0	0	0	0		0	0
	0	+	0	+	0	+		0	0
	0	+	0	+	0	+		0	—
	0	+	+	0	0	+		0	+
	0	+	+	+	0	0		0	0
	0	0	+	0	+	0		+	0
	0	0	+	0	0	+		0	0
	0	0	+	0	0	+		0	0
	0	0	+	+	+	+		+	0
	0	0	+	+	0	0		0	0
	0	0	0	+	0	+		—	—
	0	0	0	0	0	+		+	+
total	6	8	9	7	4	10		4	3

— aphids died.

Fifty-two aphides failed to cause infection on any plant.

With beet yellows virus the increase in the number of aphides to exhibit infectivity continued for the first 6 hr. of consecutive feeding, but the number of plants infected at each transfer decreased after the first 3 hr. because the aphides which first became infective ceased to infect, or infected at longer and longer intervals. Infectivity was higher again at the sixth transfer, as with the non-persistent viruses on the last of their consecutive plants, because the aphides were given longer test feeding time.

Had short i.f. times been given it is obvious that the distribution of infection with beet yellows virus would have been entirely different from that obtained with beet mosaic and the non-persistent viruses; preliminary fasting does not affect vector efficiency with beet yellows virus (see p. 204), and the effect of short i.f. on the infectivity of non-persistent viruses depends upon the preliminary fasting treatment.

When the effect of preliminary fasting is not involved there seems, superficially, to be no essential difference between the transmission of beet yellows and beet mosaic

virus, except in the time taken to acquire and disseminate infection and the number of plants infected. When there has been preliminary fasting the transmission of the two types of virus is entirely different. Thus one of the essential differences between them lies in the special property which makes beet mosaic and the other non-persistent viruses susceptible to the effect of preliminary fasting.

III. EFFECT OF POST-INFECTION FASTING

With Hy 3, potato Y and tobacco etch viruses, infectivity was retained longer by the vectors when fasting after infection feeding than when feeding, and the optimum retention of infectivity during post-infection fasting was by aphides which had received preliminary fasting and short I.F. Experiment 5 shows the effect of these treatments on the transmission of beet mosaic virus.

Experiment 5 (table 6). Beet mosaic virus

Treatments—all combinations of:

- (1) Two preliminary fasting times: 0, 4 hr.
- (2) Three infection feeding times: 2 min., 1, 16 hr.
- (3) Four post-infection fasting times: 0, 1, 5, 20 hr.

Constant: test feeding time, 20 hr.

Aphides: 1 per plant.

Plants: 5 per treatment, 4 replications.

TABLE 6. EFFECT OF VARYING TIMES OF PRELIMINARY FASTING, INFECTION FEEDING AND POST-INFECTION FASTING ON TRANSMISSION OF BEET MOSAIC VIRUS BY *M. PERSICAE*

preliminary fasting times	infection feeding times	post-infection fasting times				
		0	1 hr.	5 hr.	20 hr.	total
none	2 min.	6	3	2	1	12 (b)
	1 hr.	3	5	1	0	9
	16 hr.	16	10	8	3	37
	total	25 (a)	18	11	4	58 (d)
4 hr.	2 min.	18	7	2	4	31 (b)
	1 hr.	7	4	3	1	15
	16 hr.	17	10	10	6	43
	total	42 (a)	21	15	11	89 (d)
total		67 (c)	39	26	15	147

Standard errors: (a) ± 1.77 , (b) ± 2.05 , (c) ± 2.51 , (d) ± 3.55 .

The effects of varying preliminary fasting and I.F. periods were the same as in experiments 1 and 2. As in experiment 2 the number of plants infected increased with increasing I.F. for both times of preliminary fasting. The infections decreased with increasing post-infection fasting time, and the rate of decrease was most rapid for the first few hours of fasting.

Beet mosaic virus was retained by the fasting vectors longer than the other non-persistent viruses. 22 % of the initially infective aphides were still infective after 20 hr. fasting. Previously Hy 3 had survived in the fasting vector longest of the non-persistent viruses, but less than 3 % of the aphides retained infectivity for even 12 hr. (Watson 1938). This compares very unfavourably with the performance of the same vectors with beet mosaic virus.

Beet yellows virus survives in the feeding vectors for several days, and it might be supposed that it would be lost even more slowly from the fasting vectors, since feeding vectors presumably lose virus by ejaculation into the healthy plants. Experiments 6 and 7 show that this is not so.

Experiment 6 (table 7). Beet yellows virus

Treatments—all combinations of:

(1) Three infection feeding times: $\frac{1}{2}$, 3, 20 hr.

(2) Three post-infection fasting times: 0, 3, 20 hr.

(3) Three test feeding times: $\frac{1}{2}$, 3, 20 hr.

Aphides: 5 per plant.

Plants: 5 per treatment, 8 replications.

TABLE 7. EFFECT OF POST-INFECTION FASTING WITH VARYING INFECTION AND TEST FEEDING TIMES. BEET YELLOWS VIRUS

test feeding times	infection feeding times	post-infection fasting times			
		0	3 hr.	20 hr.	total
$\frac{1}{2}$ hr.	$\frac{1}{2}$ hr.	4 (a)	1	5	10 (b)
	3 hr.	13	14	6	33
	20 hr.	23	12	7	42
	total	40 (b)	27	18	85 (c)
3 hr.	$\frac{1}{2}$ hr.	5	8	3	16
	3 hr.	33	18	8	59
	20 hr.	34	24	16	74
	total	72	50	27	149
20 hr.	$\frac{1}{2}$ hr.	7	3	4	14
	3 hr.	24	19	9	52
	20 hr.	35	28	11	74
	total	66	50	24	140

Standard errors: (a) ± 3.13 , (b) ± 5.42 , (c) ± 9.89 .

The number of plants infected increased with increasing I.F. and test feeding times as in previous experiments. Infectivity was lost unexpectedly rapidly during post-infection fasting. Infectivity decreased by 34 % during the first 3 hr. of fasting, although if the aphides had fed on healthy plants for 3 hr. their infectivity at a subsequent feeding would have been higher than their initial infectivity.

There was no indication that when the aphides were allowed to feed again after fasting they recovered any of the infectivity which they had lost, for the difference between the performances of aphides which had fasted and those which had not fasted was about the same for 30 min. test feeding as for 20 hr. If the aphides recovered infectivity during feeding on the test plants, the decrease in infectivity with increasing post-infection fasting time would have been smaller for 20 hr. test feeding than for 30 min.

The decrease in the number of plants infected caused by increasing the post-infection fasting time was slower when the I.F. time was short than when it was long. This appeared in the analysis of variance as a highly significant interaction between I.F. and post-infection fasting time. Thus the rate of loss of infectivity during fasting seems to depend to some extent on the aphides' initial content of active virus, and is slower when this quantity is small than when it is large.

Experiment 7 was made with beet yellows virus to compare directly the effect of post-infection fasting with that of feeding on intermediate healthy plants.

Experiment 7 (table 8). Beet yellows virus

Treatments—all combinations of:

- (1) Post-infection fasting and feeding on intermediate healthy leaves.
- (2) Six times of post-infection fasting or intermediate feeding: 0, 1, 2, 5, 10, 20 hr.

Constant: I.F. time 24 hr.

Test feeding time: 2 hr.

Aphides: 3 per plant.

Plants: 10 per treatment, 7 replications.

TABLE 8. COMPARISON OF EFFECTS OF POST-INFECTION FASTING AND FEEDING ON INTERMEDIATE HEALTHY PLANTS, FOR VARYING TIMES. BEET YELLOWS VIRUS

treatment of aphides after I.F.*	times of feeding or fasting						
	0	1 hr.	2 hr.	5 hr.	10 hr.	20 hr.	total
feeding	33 (a)	37	44	32	18	13	177 (b)
fasting	31	27	23	14	10	7	112

Standard errors: (a) ± 4.66 , (b) ± 7.98 .

* Test feeding time = 2 hr.

The number of plants infected decreased rapidly during the first 5 hr. of post-infection fasting, but there was no loss of infectivity during the corresponding period of feeding on intermediate healthy leaves. Instead, more plants were infected after 2 hr. of intermediate feeding than after 0 or 1 hr., and after 5 hr. intermediate feeding the aphides still maintained their initial level of infectivity. This has also been found to occur when the intermediate feeding period was spent on *Hyoscyamus niger* which is not susceptible to beet yellows virus; so that the maintenance of the infectivity of the aphides is due merely to the fact that they

have been feeding and not to reinfection, or to the virus being kept active in a medium consisting of susceptible plant sap. After the first 5 hr. of treatment the rate of loss of infectivity from fasting aphides became slower, and that from the feeding aphides faster, so that eventually the rates of loss were about the same. It is known that feeding aphides would continue to lose infectivity slowly for about another 48 hr. (Watson 1940), but it would not be practicable to test the loss from fasting aphides over this period as their general condition would deteriorate and they would not be comparable with the feeding aphides.

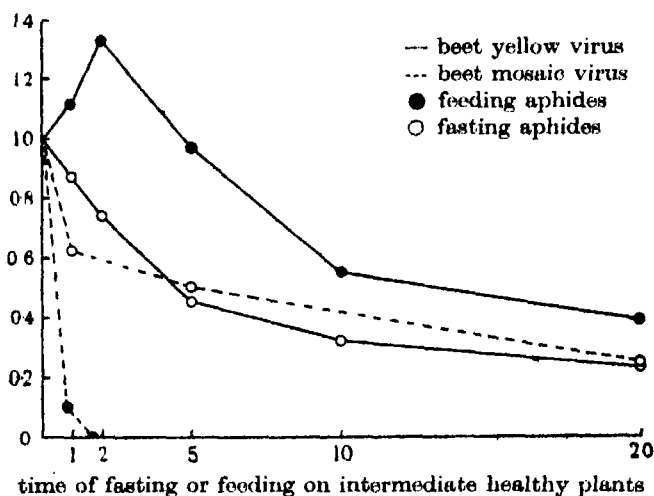


FIGURE 1. Changes in the ability of *M. persicae* to transmit beet yellows and beet mosaic viruses, after varying feeding and fasting treatments. The solid lines refer to beet yellows and the dotted lines to beet mosaic virus. The points marked by solid circles show the infectivity of aphides which fed between I.F. and test feeding, and the open circles show the infectivity of aphides which fasted between I.F. and test feeding.

Beet yellows virus thus differs greatly from beet mosaic and the other non-persistent viruses in that the number of plants infected decreases more rapidly when the vectors fast than when they feed; with all the non-persistent viruses tested, infectivity is lost much more rapidly by the feeding than by the fasting vectors. This difference in behaviour in response to treatment of the vector is shown graphically in figure 1, for beet mosaic and beet yellows viruses. The data used are taken from tables given in this paper.

In figure 1 the infectivity of the aphides after varying post-infection fasting and feeding treatments is expressed as a fraction of their infectivity immediately after cessation of I.F. The I.F. times were 16–24 hr., and the test feeding times were 2 hr. for the beet yellows experiments; 50 min. for the beet mosaic vectors which were given post-infection feeding, and 20 hr. for those given post-infection fasting, as no short test feeding figures were available for this treatment.

The behaviour of the two viruses in the feeding aphides is very different. Infectivity with beet mosaic virus was lost during the first hour of feeding, while that

with beet yellows virus was only reduced by 50 % during 20 hr. of feeding. In the fasting aphides, however, their behaviour was similar, for both retained about 20 % of their infectivity after 20 hr. fasting. The loss from the fasting vectors of beet mosaic during the first hour of fasting was more rapid than that of beet yellows.

The behaviour of beet mosaic virus during post-infection fasting is more consistent than that of beet yellows virus. In experiment 6, the changes in infectivity due to the varying treatments were about the same on each occasion, hence the smallness of the errors. In experiment 7 with beet yellows virus, and also in experiments 8 and 9, the losses during the first few hours of fasting were very variable, sometimes large, and sometimes inconsiderable, hence the errors were much larger in these experiments than in the beet mosaic virus experiments. It is possible that the variation in rate of loss of beet yellows virus from the vectors was due to variations in glasshouse temperatures. Kassanis (1941) showed that loss of severe etch virus from the fasting vectors was much slower when the insects were held at low temperatures, and beet yellows virus is more sensitive to changes in temperature than any of the non-persistent viruses (Kleczkowski & Watson 1944).

Experiment 8 shows that a period of feeding given before the post-infection fasting treatment does not prevent rapid loss of infectivity during subsequent fasting; i.e. the introduction of healthy sap into the vectors does not prevent their losing infectivity unless feeding is maintained. Also there is no recovery of the infectivity lost during fasting, detectable in two $1\frac{1}{2}$ hr. feeding periods given after the fasting treatment.

Experiment 8 (table 9). Beet yellows virus

Treatments—all combinations of:

(1) Aphides fed for $1\frac{1}{2}$ hr. on healthy leaves immediately after I.F. (before post-infection treatment), and aphides not fed after I.F.

(2) 12 hr. post-infection fasting and 12 hr. intermediate feeding.

Constant: I.F. time, 24 hr.; two consecutive test feedings of $1\frac{1}{2}$ hr. each.

Aphides: 2 per plant.

Plants: 10 per treatment, 5 replications.

TABLE 9. EFFECT OF CONSECUTIVE FEEDING AFTER POST-INFECTION FASTING, AND OF FEEDING ON HEALTHY PLANTS BEFORE POST-INFECTION FASTING

treatments		infections obtained at two successive $1\frac{1}{2}$ hr. test feedings		
		A	B	total
post-infection fasting—12 hr.	$1\frac{1}{2}$ hr. feeding between I.F. and fast	11	12	23
	no feeding between I.F. and fast	17	8	25
	total	28	20	48
intermediate feeding—12 hr.	$1\frac{1}{2}$ hr. feeding between I.F. and intermediate feeding	19	18	37
	no feeding between I.F. and intermediate feeding	20	19	39
	total	39	37	76

Fewer plants were infected when the aphides fasted before test feeding than when they fed; a period of feeding given before the post-infection fasting or feeding treatment slightly reduced the number of plants infected, but did not affect the loss due to fasting. As there was no increase in infectivity between the first and second successive test feedings given after the fasting or feeding treatments, the long fasting period did not merely delay the increase in infectivity of the aphides which normally occurs during the first few hours of test feeding, but eliminated it.

IV. THE SEPARATION OF BEET YELLOWS AND BEET MOSAIC VIRUSES FROM A MIXED INFECTION IN THE SAME HOST PLANT, BY DIFFERENTIAL FASTING AND FEEDING TREATMENTS OF THE VECTORS

Viruses with the same vectors and host range are sometimes difficult to isolate from the complexes in which they occur in nature. Experiment 9 shows the use of differential fasting and feeding treatments in separating such complexes, and it also shows that the behaviour of the viruses is substantially the same with *A. fabae* (Scop.) as the vector, as it is with *M. persicae* (Sulz.), although *A. fabae* is a less successful vector. The sources of infection were young sugar-beet plants infected by aphides at the same time with beet mosaic and beet yellows viruses.

Experiment 9 (table 10). Beet mosaic and beet yellows viruses vectors M. persicae and A. fabae

Treatments—all combinations of:

- (1) Vectors: *M. persicae* and *A. fabae*.
- (2) Two preliminary fasting times: 0, 16 hr.
- (3) Three infection feeding times: 5 min., 1, 24 hr.

Constant: Four consecutive test feeding times, A 10 min., B 2 hr., C 24 hr., D 24 hr.

Aphides: 2 per plant.

Plants: 5 per treatment, 6 replications.

The separation of the viruses was very successfully accomplished, as only three plants became infected with both viruses at once. With beet mosaic virus, both *M. persicae* and *A. fabae* caused an optimum number of infections after preliminary fasting and short I.F. These infections occurred during the first 10 min. of feeding on the consecutive healthy plants. A second optimum occurred after 24 hr. I.F., and most of the infections with beet mosaic virus were again on the first plants. With beet yellows virus very few plants became infected when the I.F. time was short; after 24 hr. I.F. a few of the second and many of the third plants became infected. Beet mosaic was never carried to the third plants, but after 24 hr. I.F. a few of the second plants became infected, and mixed infections occurred.

In practice the separation of unknown viruses from a complex does not require so elaborate a technique as that used in experiment 9, which was designed to show

how the method works, and to compare the performance of the two aphides, as well as to separate the viruses. The only treatment necessary would be preliminary fasting of 12-16 hr. (overnight), and two infection feeding times—2-5 min. and 24 hr.; followed by consecutive feedings of which the first must be short (about 10 min.), and the second 2 or 3 hr., in order to eliminate any non-persistent virus from the aphides. The length of the later consecutive feedings would depend upon the viruses to be isolated, for the period of delay in production of optimum infectivity during test feeding time varies very much with different persistent viruses. For instance, with beet yellows virus it is only a few hours, but with other persistent viruses it may be many hours, or even days, before the optimum number of infections is obtained, and with these viruses infectivity of the vectors is at first absent or negligible. After some hours' feeding, vector efficiency with a virus like beet yellows is much reduced, and the virus with the longer period of delay in the production of optimum infectivity would be obtained alone.

TABLE 10. SEPARATION OF BEET MOSAIC AND BEET YELLOWS FROM A MIXED INFECTION, BY DIFFERENTIAL TREATMENT OF THE VECTORS

infection feeding time	vector	virus	no preliminary fasting consecutive infections					16 hr. preliminary fasting consecutive infections				
			A	B	C	D	total	A	B	C	D	total
5 min.	<i>M. persicae</i>	mosaic	3	1	0	0	4	15	2	0	0	17
		yellows	0	0	1	0	1	0	0	0	0	0
	<i>A. fabae</i>	mosaic	1	0	0	0	1	3	1	0	0	4
		yellows	0	0	0	0	0	0	0	0	0	0
1 hr.	<i>M. persicae</i>	mosaic	1	0	0	0	1	2	1	0	0	3
		yellows	1	3	1	0	5	0	2	1	0	3
	<i>A. fabae</i>	mosaic	0	0	0	0	0	0	0	0	0	0
		yellows	0	1	0	0	1	1	0	0	0	1
24 hr.	<i>M. persicae</i>	mosaic	4	1*	0	0	5	6*	2*	0	0	8
		yellows	2	16	17	6	41	4	18	22	6	50
	<i>A. fabae</i>	mosaic	0	0	0	0	0	1	0	0	0	1
		yellows	0	3	4	1	8	0	5	5	2	12

Consecutive feeding times: A, 10 min.; B, 2 hr.; C, 24 hr.; D, 24 hr.

* The figures for mosaic and yellows are determined from the same set of plants. At * one plant became infected with both viruses and therefore appears as both mosaic and yellows.

Probably the most effective treatment would be to give two 24 hr. periods for the third and fourth consecutive feedings, as was done in experiment 8; then, if the infection obtained were still suspected of being a complex, to use this isolate in further consecutive experiments giving three or four longer or shorter consecutive feeding periods, according to where the infection was obtained in the previous experiment.

Thus the method could be used for separating different persistent viruses from each other as well as persistent from non-persistent viruses, though it would not

necessarily be successful in separating non-persistent viruses from each other, as their behaviour in the feeding vectors is too similar. They might be separable by differential post-infection fasting treatments of the aphides.

DISCUSSION

When the aphides transmitting beet mosaic virus are made to fast before feeding on a source of infection they infect the greatest number of plants if they are given only 2 or 3 min. I.F. Their infectivity decreases by about 60 % during the next hour of I.F. and increases again, sometimes to its initial level, when the I.F. time is increased to several hours. When there has been no preliminary fasting the infectivity of the aphides remains constant at a low level for the first 2 or 3 hr. I.F., and then increases at about the same rate as it does with aphides which have fasted before I.F.

After preliminary fasting and short I.F. a vector can transmit beet mosaic virus to several healthy plants in successive 5 min. test feedings, but the probability of infection decreases at each transfer, and infectivity of the aphides is exhausted after about the sixth transfer, that is, about 35 min. from the cessation of I.F. In these conditions most of the aphides infect the first plants on which they feed. When the aphides are given several hours' I.F. fewer of them become infective, and fewer plants are infected by each individual. The infectivity of the aphides as a group, however, declines less rapidly than it does after preliminary fasting and short I.F., because some of them do not infect the first plants on which they feed but become infective at progressively later feedings. In other words, the number of aphides exhibiting infectivity increases with increasing time of feeding on healthy plants, although the total number of plants infected still decreases at each successive transfer.

When aphides fast after I.F. infectivity is lost much more slowly than when they feed on healthy plants. After fasting for 20 hr. vectors of beet mosaic virus retained, on the average of all previous treatments (preliminary fasting and infection feeding times), about 20 % of their initial infectivity. The loss of infectivity from aphides given 1 hr. I.F. was slightly more rapid during fasting than when they had been given very short or very long I.F. time.

On the whole this behaviour of beet mosaic virus in relation to its vectors accords with that of the sap-transmissible, aphid-transmitted viruses which have been called the non-persistent group. The other viruses which have been found to exhibit these properties are: *Hyoscyamus* 3, potato Y, cucumber 1, severe etch of tobacco, soya bean mosaic, and common pea mosaic.

The ability of vectors to transmit beet yellows virus is not affected by preliminary fasting. Infectivity always increases with increasing I.F. time from a very low level, detectable after about 5 min. I.F., to a maximum somewhere between 10 and 20 hr. The number of plants infected also increases with increasing feeding time on the healthy plants, reaching a maximum after about 8 hr. of feeding. Infectivity

does not increase, but generally decreases if the aphides fast instead of feeding after cessation of I.F. The increase in infectivity with increasing test feeding time occurs whatever the I.F. time, but when the I.F. time is short few aphides are capable of causing infection at any time, and in most experiments no infections are obtained during the first hour of test feeding. Prolonging the I.F. time greatly increases the vector efficiency of the aphides, and some are able to cause infection during the first hour of feeding, though there are still more which infect later.

In most experiments which are designed to show the presence of an 'incubation period' of a virus in a vector, the I.F. times used are very short, because it is believed that long I.F. times would 'mask' the 'incubation period'. But with some of these viruses the I.F. time necessary to produce optimal infectivity of the vectors is very much longer than the 'incubation period'. The experiments therefore are made in conditions in which the vectors are only weakly infective, and the chances of their causing infection soon after cessation of I.F. are very small, or negligible. It is evident from the data published for many of these viruses that, even when the I.F. time is many times longer than the supposed 'incubation period', a very large proportion of the vectors fail to cause infection until they have fed on the healthy plants for a period corresponding to the 'incubation period'. No really satisfactory explanation has been put forward to account for this behaviour.

There is no previous record of infectivity of a virus being lost, as is beet yellows virus, more rapidly from the fasting than from the feeding vectors. Bennett & Wallace (1938) found that vectors infective with beet curly-top virus lost infectivity almost completely when they were made to fast for about 18 hr., but the loss was only temporary, and ability to cause infection was regained during four successive 5 min. feedings on healthy plants; with beet yellows virus, after prolonged post-infection fasting, the loss seems to be permanent. The performance of the starved curly-top vectors was not directly compared with that of vectors which had fed continuously for corresponding periods, but the figures given do not suggest that any infectivity was permanently lost by the fasting vectors as with beet yellows virus. Storey (1928) used fasting as a routine treatment of infective vectors of maize streak virus disease, to induce the insects to feed rapidly and uniformly; apparently this treatment did not reduce the probability of infection though again the performance of the starved vectors was not directly compared with those that had fed. No information is available about the behaviour of other aphid-transmitted persistent viruses in the fasting vectors.

The terms 'persistent' and 'non-persistent' refer to the length of time for which the viruses remain active in the vectors. In general, vectors of persistent viruses retain infectivity much longer than those of the non-persistent viruses. But the range of time over which changes in infectivity take place is very wide, especially with the persistent viruses, whose vectors may take hours, days, weeks, or even months to acquire and lose infectivity with different viruses. Non-persistent viruses all seem to be acquired and lost rapidly, but even with these there is some variation, and there can be 'overlapping' of the survival times in the vectors as

there is with beet yellows and beet mosaic viruses. In these circumstances the terms 'persistent' and 'non-persistent' no longer retain their original significance.

There are other ways in which non-persistent viruses may resemble persistent ones in their behaviour. Infectivity with beet mosaic increases with increasing time on the infected plants when the aphides have not starved previously, or when the effect of preliminary fasting has disappeared. Also, in some circumstances, infectivity may increase with increasing feeding time on the healthy plants, though again this does not occur after preliminary fasting.

Thus the properties in which non-persistent viruses may resemble persistent viruses are those which are exhibited when the infectivity of the vectors is not being influenced by preliminary fasting.

It is unlikely that viruses, such as the non-persistent group, which respond to preliminary fasting of the vectors could be retained for long periods by the feeding vectors. Infectivity of previously fasted vectors decreases rapidly when they are feeding on a source of infection, and apparently the loss of infectivity is caused by the act of feeding of the vectors and not the virus source (Watson & Roberts 1939; Roberts 1940). Consequently, feeding on healthy plants, when there is no virus to replace that which is lost, must rapidly render the vectors non-infective. On the other hand, it is not necessarily true that all persistent viruses will be retained for long periods by their vectors. The length of time for which they are retained varies with the length of the i.f. time, i.e. with the virus content of the vectors. This has been shown for beet yellows virus, and also for beet curly-top virus (Freitag 1936; Bennett & Wallace 1938), and maize streak virus (Storey 1938). Plant hosts vary in the amount of virus which the vectors can obtain from them; for instance, aphides become more highly infective when fed on tobacco plants infected with potato virus Y, than on some varieties of potato (unpublished data), and some persistent viruses may exist in hosts which provide such a poor source of virus that the vectors would remain infective for little longer than those of the non-persistent viruses. There may also be other factors which could affect the retention of infectivity by vectors of persistent viruses, while still excluding them from the non-persistent group.

It seems, therefore, that division of the two types of viruses on the basis of the time taken for any specific reaction is not satisfactory. A more satisfactory basis, at present, would be the response to preliminary fasting. The two groups distinguished by the presence or absence of this property are probably not of equal standing; the non-persistent viruses, which possess it, appear to be a close group of aphid-transmitted viruses with similar physical properties, but the persistent viruses, which do not possess it, have many dissimilar attributes. They have vectors from different families, or even orders, of insects; their sap transmissibility varies greatly, and they respond differently to some treatments of their vectors; for instance, beet yellows and beet curly-top viruses respond differently to post-infection fasting of the vectors. There are possibly many subgroups among the persistent viruses each equivalent to the non-persistent group. Dandelion yellow

mosaic virus (Kassanis 1944), appears to be 'non-persistent', for it survives in the feeding vector for less than 1 hr., but the vectors require at least 3 hr. to become infective even when previously starved, and so the virus does not belong to the 'non-persistent' group as it is here defined.

There is some doubt as to whether such properties as those described in this paper will ever provide really satisfactory bases of classification for the insect-transmitted viruses (Bawden 1943), but the distinctions have a useful application. The variation in response to preliminary fasting and feeding time on the healthy plants can be used in experiments to separate persistent and non-persistent viruses from complexes in which they may exist; the results of such experiments will also give some information about other properties of the viruses isolated.

The general use of routine experiments, such as that described in § IV, would help to unify the results obtained by different workers, but to be of any value they should be carried out with sufficient replications to give reasonably small errors, as variation in experimental conditions causes great variation in the ease with which viruses are transmitted.

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The biological activity of phenolic compounds.

The effect of surface active substances upon the penetration of hexyl resorcinol into

Ascaris lumbricoides var. *suis*

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The effect of surface active substances (sodium cholate, sodium oleate and cetyl trimethyl ammonium bromide (C.T.A.B.)), upon the penetration of hexyl resorcinol into the pig round-worm (*Ascaris lumbricoides* var. *suis*) has been measured quantitatively, and parallel measurements carried out upon the interfacial activity of these solutions against an inert mineral oil.

In all three cases a marked similarity of action, differing only in degree, was observed. Using a fixed hexyl resorcinol concentration (0.025 %) the soap in dilute solution accelerated the penetration of this drug, the maximum accelerations being in the order sodium cholate < sodium oleate < C.T.A.B., but when present in high concentration the penetration was completely inhibited.

The soaps alone were found to penetrate *Ascaris* very slowly, if at all, the amounts taken up being reasonably explained on the basis of surface adsorption only.

Measurement of interfacial tensions showed that the soap and hexyl resorcinol molecules associate at an interface, forming a labile complex of increased surface activity. Using a fixed drug concentration (0.025 % as above), the interfacial tension went through a minimum as the soap concentration was increased, ultimately rising to the value for the soap alone. Maximum interfacial activity (i.e. minimum interfacial tension), was found in all cases to occur at that soap concentration giving maximum hexyl resorcinol penetration into *Ascaris*, and was in the same order, viz. cholate < oleate < C.T.A.B. The interfacial tension measurements also showed that this particular soap concentration corresponded to the onset of micellar aggregation of the soap molecules.

From the interfacial tension data a simple explanation for the biological measurements can be suggested, which at the same time appears to explain a number of observations upon other biological systems, in particular the effect of soaps upon the bactericidal activity of phenols.

This explanation suggests that the biological activity of hexyl resorcinol (as measured by its rate of penetration) is determined by the interfacial activity of the mixture, and that when soap micelles are present the drug distributes itself between the micelles and any other interface present (e.g. oil/water or *Ascaris*/water). The maximum biological activity thus occurs at the critical concentration for micelle formation, since this has maximum interfacial activity.

At high soap concentrations the hexyl resorcinol is mainly held by the soap micelles, so that the biological activity is thereby diminished, ultimately to zero since the soaps alone penetrate so extremely slowly.

In the case of C.T.A.B./hexyl resorcinol mixtures it has been possible, from physical data only, to calculate a theoretical curve for the biological activity which is in good agreement with experiment.

The possible bearings of these conclusions upon *in vivo* activity of hexyl resorcinol as an anthelmintic, and upon drug action in general, are briefly mentioned.

During an investigation into the anthelmintic action of aqueous solutions of hexyl resorcinol (Trim 1944), certain effects were produced by bile salts which could not be satisfactorily explained. In particular it was found that, for a fixed hexyl resorcinol concentration (0.025 %), increasing the concentration of bile salt

(sodium cholate) reduced the rate of penetration of the drug into the nematode worm *Ascaris lumbricoides* var. *suis*, the inhibition becoming effectively complete at 0.5 % sodium cholate.

In order to investigate the problem further, measurements of the interfacial activity of the bile salt/hexyl resorcinol systems against an inert hydrocarbon oil were carried out; an oil/water interface being chosen rather than say, air/water, since the surface of *Ascaris* is known to be hydrophobic. These measurements suggested a very simple explanation of the inhibition and the development of the work showed that the effects obtained with bile salts could be related to those given by other detergents such as sodium oleate and ecetyl trimethyl ammonium bromide (a synthetic cationic detergent referred to below as C.T.A.B.). These latter compounds also inhibited hexyl resorcinol penetration when present in high concentrations, but differed from bile salt in showing a pronounced accelerating effect when sufficiently diluted, thus indicating one possible mode of increasing the rate of penetration of the drug into *Ascaris*.

EXPERIMENTAL

The interfacial tensions between nujol (a pure, high-boiling paraffin similar to medicinal paraffin) and the physiological aqueous media were measured by means of the ring method, absolute values being obtained by applying the corrections of Harkins & Jordan (1930). Measurements were made at room temperature (18–20° C).

The penetration of hexyl resorcinol at 37°C was followed by the method described by Trim (1944), a micro-colorimetric method using the well-known azo-dye reaction. *p*-Amino-benzoic acid was diazotized by the rapid and simple method devised by Dangerfield, Gaunt & Wormall (1938). Small aliquots of the hexyl resorcinol solution were coupled with the resulting diazonium salt in aqueous solution in the presence of strong soda to give an intense orange-red solution of azo dye which was measured in a direct vision colorimeter. The colour obeyed Beer's law and quantities of hexyl resorcinol down to 5–10 µg. could be measured with ease and accuracy.

Cholate was measured by the micro-colorimetric method of Ohiyama (1938) which is based on the measurement of a carmine-red colour slowly developed by the reaction between laevulose and the bile acid in the presence of concentrated hydrochloric acid.

C.T.A.B. was also measured colorimetrically by the application of a general method for cationic detergents introduced by Auerbach (1943), which was found to be suitable. The detergent solutions were shaken with brom-phenol-blue and dilute sodium carbonate in the presence of ethylene dichloride which quantitatively extracts the coloured salt so formed between the C.T.A.B. and brom-phenol-blue molecules. The ethylene dichloride solution was separated and cleared by shaking with a small flake of sodium hydroxide and the colour measured in a direct-vision colorimeter against a suitable standard. The presence of hexyl resorcinol changed

the tint and intensity of the colour, but, provided it was arranged to be present in the same concentration in the standard and unknown solutions, did not interfere with the determinations.

Hexyl resorcinol also interfered with the cholate colour reaction but was readily removed by extracting the aqueous solutions three times with equal volumes of a 1:1 mixture of benzene and light petroleum (40–60°).

RESULTS

Sodium cholate

Experiments with this substance were conducted with solutions in the physiological saline described by Baldwin (1943) at pH 6.6. Figure 1 gives the interfacial tension-concentration curve, and also the biological activity as measured by the amount of hexyl resorcinol (mg./g. wet-weight worm) penetrating *Ascaris* in

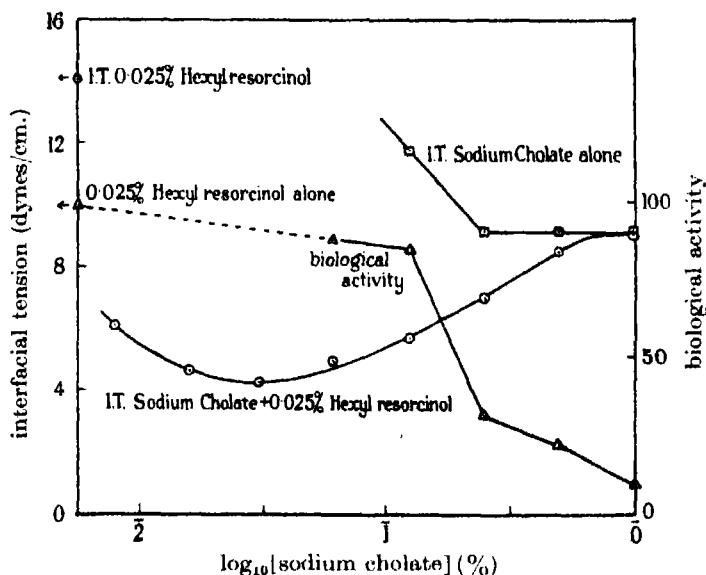


FIGURE 1. Sodium cholate/0.025 % hexyl resorcinol mixtures. Interfacial tension against nujol and biological activity measured by the penetration of hexyl resorcinol as percentage of control (0.025 % hexyl resorcinol alone).

20 min., for a fixed hexyl resorcinol concentration (0.025 %) and cholate concentrations ranging from 0.008 to 1.0 %. The interfacial tension curve for the cholate alone is included for comparison.

Measurements of the concentration of cholate in the medium made while following the uptake of drug, and also in the absence of drug, showed no sensible removal of the bile salt by the worms over the usual period of the experiment (1 hr.), although more prolonged incubation of the worms with bile-salt solutions did lead to a significant depletion. The cholate concentrations used were 0.2, 0.1 and 0.031 %; the hexyl resorcinol concentration 0.025 % in all cases.

Sodium oleate

Sodium oleate resembles sodium cholate in inhibiting hexyl resorcinol penetration at high concentrations, but differs from it in producing a marked acceleration in dilute solutions, the maximum accelerating effect observed being about 300 % at about 0.05 % oleate. The effects of oleate concentration upon the rate of penetration of hexyl resorcinol into *Ascaris*, together with the interfacial tension data, are given in figure 2, the hexyl resorcinol concentration being fixed at 0.025 % as before. The aqueous medium was 0.9 % NaCl, pH 6.5, in place of the modified Ringer's solution, in order to avoid precipitation of the oleate by calcium ion.

Sodium oleate, however, suffers from certain inherent disadvantages such as sensitivity to traces of calcium and heavy metal cations, and the formation of acid soaps. Consequently a modern synthetic detergent free from these troubles was chosen for investigation.

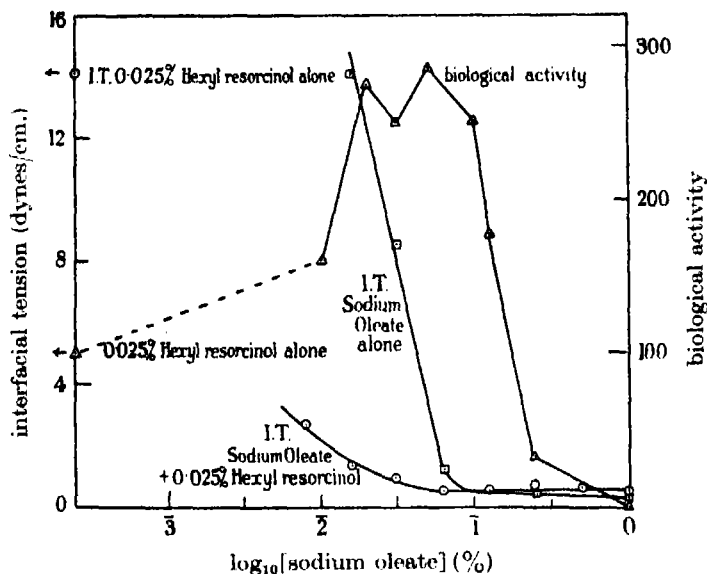


FIGURE 2. Sodium oleate/0.025 % hexyl resorcinol mixtures. Interfacial tension against nujol and biological activity measured by the rate of penetration of hexyl resorcinol as percentage of control (0.025 % hexyl resorcinol alone).

Cetyl trimethyl ammonium bromide (C.T.A.B.)

This substance is freely soluble in cold water and essentially unaffected by pH or metallic cations. Consequently it provides a system suitable for elucidating the basic physico-chemical factors involved. The biological and interfacial tension data, given in figure 3, show marked similarity to those given by sodium oleate, the maximum acceleration being somewhat greater (ca. 350 % at ca. 0.004 % C.T.A.B.).

Auerbach's (1943) method for estimating cationic detergents, as outlined above, was used to follow changes of C.T.A.B. concentration in the solutions used. Both

with and without hexyl resorcinol the initial rate of depletion of C.T.A.B. from the solutions in the presence of worms was fairly rapid, but fell off sharply, and the extent of depletion was sensibly the same in both cases. In figure 4 the uptake of hexyl resorcinol and the depletion of C.T.A.B. from a solution containing 0.025 % hexyl resorcinol and 0.004 % C.T.A.B. are plotted in molecular quantities for comparison. It is clear that with or without C.T.A.B. the rate of penetration of hexyl resorcinol exceeds that of C.T.A.B. many times. (The acceleration produced by C.T.A.B. in figure 4 is seen to differ somewhat from that shown in figure 3, owing to the use of a different sample of C.T.A.B.)

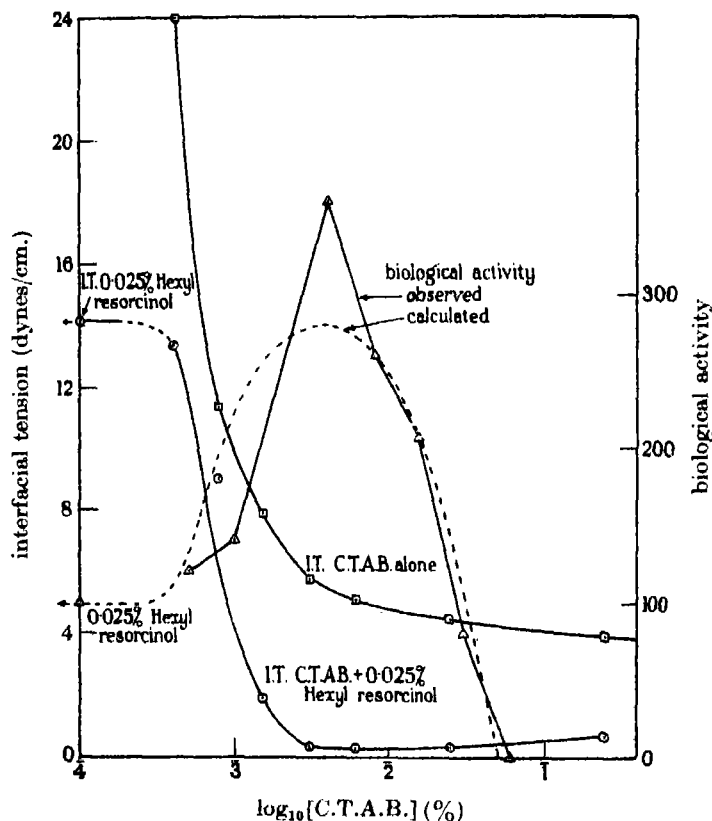


FIGURE 3. C.T.A.B./0.025 % hexyl resorcinol mixtures in 0.9 % NaCl. Interfacial tension against nujol and biological activity measured by the penetration of hexyl resorcinol as percentage of control (0.025 % hexyl resorcinol alone).

The rate and the extent of depletion of C.T.A.B. were also measured at 0.1 % concentration, where the penetration of hexyl resorcinol from 0.025 % solutions is completely inhibited. It was found that the depletion of C.T.A.B. was no greater than at the lower accelerating concentration used above. Consequently the possibility that the inhibition of hexyl resorcinol penetration arises from a blocking of the worm's surface by adsorbed soap micelles must be ruled out. (See also the discussion below.)

The rapidity of the initial C.T.A.B. uptake and the slowness of any further depletion suggests that only adsorption on to the worm's surface is occurring. On this hypothesis the area occupied per molecule can readily be calculated, giving a value of ca. 1.4 \AA^2 , i.e. about fifteen times smaller than the value of 20 \AA^2 for a close-packed monomolecular film. (The depletion was taken as $1 \times 10^{-7} \text{ g.mol./g.}$ of worm, and the geometrical surface area of the worms was measured and found to be ca. $8.5 \text{ cm.}^2/\text{g.}$)

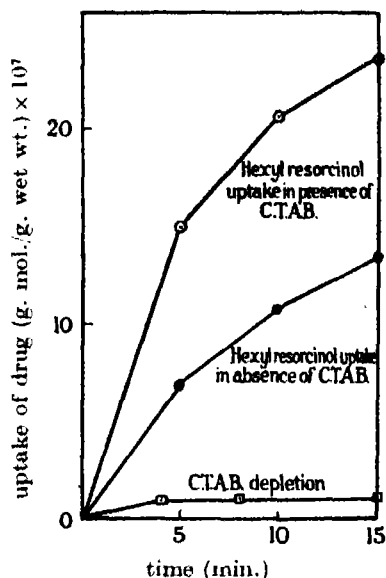


FIGURE 4. Removal by *Ascaris* of hexyl resorcinol from 0.025 % solution, alone and in the presence of 0.004 % C.T.A.B., and of C.T.A.B. from the latter solution.

Whilst a certain part of this apparent discrepancy may be due to a true penetration into *Ascaris*, it is certain that the greater part can be ascribed to the difference between the geometrical and the true surface areas. Morphologically the surface of *Ascaris* is known to consist of a series of microscopic ridges which would increase the surface area considerably, and added to this there may well be a submicroscopic structure of further complexity. Clearly no final decision can yet be given on this point, but in view of its importance some further examination is indicated.

Hexylresorcinol

Figure 5 shows the uptake of hexyl resorcinol from solutions of different concentration, ranging from 0.05 to 0.0033 %, replotted in figure 6 to show the variation of initial penetration rate with concentration. Figure 6 also includes the interfacial tension data. The highest concentration used (0.05 %) is slightly less than the maximum solubility in water at room temperature.

Values for the adsorption of hexyl resorcinol, calculated from the Gibbs equation, increase steadily with concentration, showing that micellar aggregation is not

appreciable in the range studied (Alexander 1942*a*). At 0.05 % the adsorbed molecule occupies an area of *ca.* 33 Å², indicating approximately close packing in the interface, since the value for condensed monolayers of homologous resorcinols is *ca.* 28 Å² (Adam 1928).

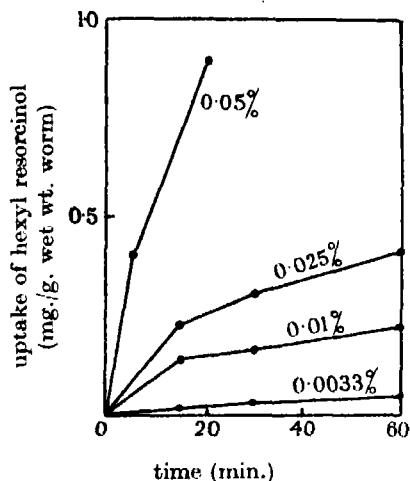


FIGURE 5. Variation of hexyl resorcinol uptake by *Ascaris* with drug concentration (0.05–0.0033 %).

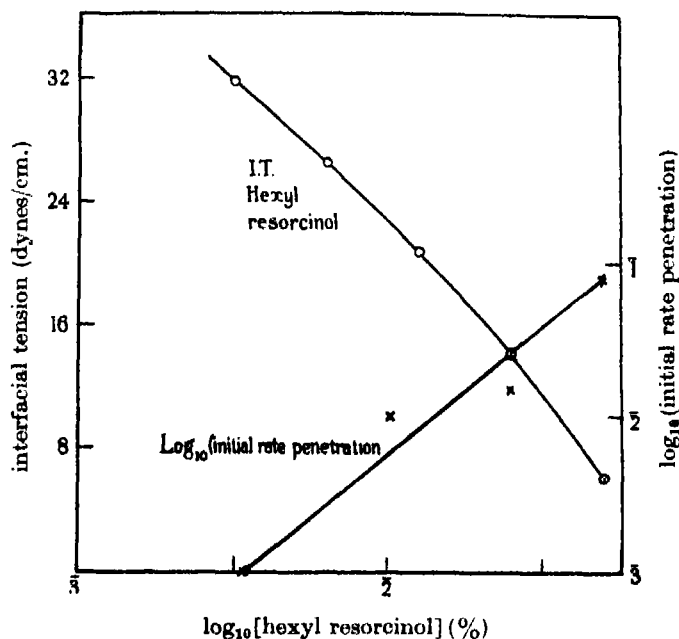


FIGURE 6. Variation of interfacial tension, and of initial penetration rate into *Ascaris*, with hexyl resorcinol concentration (0.05–0.0031 %).

DISCUSSION

It is now generally agreed that the interfacial tension of soap solutions remains sensibly constant as long as micelles are present. (The term 'soap' will be used in the following discussion to cover both natural and synthetic detergents.) When a certain critical concentration (or region of concentrations) has been passed by dilution, micelles no longer exist and the tension rises rapidly. Consequently, interfacial tension measurements provide a very simple method of determining the critical micellar concentration and of showing how this is affected by additives such as salts, hexyl resorcinol, etc.

When two surface active substances are present together these measurements also indicate whether the two components tend to associate at the interface. If they do so the observed tension will be lower than that of the more active component. Much work on these lines has been done by Schulman and co-workers, using an insoluble monolayer as one component. (For a review see Schulman 1939.) Such a labile interfacial association is generally referred to as a 'complex', and the importance of 'complex' formation in biological activity has been indicated by Schulman & Rideal (1937) in their study of haemolysis and agglutination.

In the three hexyl resorcinol/soap systems described above, there is a parallel behaviour differing only in degree. The principal results are set out in table 1 and figure 7, which is a general diagram for the purpose of simplifying the discussion.

The principal points of table 1 and figure 7 can be outlined as follows.

As the soap concentration is increased from zero the rate of penetration of hexyl resorcinol into *Ascaris* rises from its initial value *A* to a *maximum* at *B*.

Over this range of soap concentrations the interfacial tension falls to a *minimum* at *E*. (The concentration at *E* is seen to be slightly less than that at *B*. This is because the interfacial tension measurements refer to room temperature, and the biological results to 37° C. The critical concentration for micelles is lowered by reduction of temperature, and the above differences are of the order expected.)

The maximum *acceleration* produced by the soap, as indicated by the difference between *B* and *A*, is in the order cholate \ll oleate $<$ C.T.A.B., the numerical values being 0, 300 and 350 % respectively.

The maximum *lowering* of the interfacial tension (at *E*), is also in the order cholate \ll oleate $<$ C.T.A.B., the numerical values being 4.2, 0.5 and 0.3 dynes/cm. respectively.

The minimum interfacial tension of the hexyl resorcinol/soap mixture is markedly lower than that of either of its constituents, indicating 'complex' formation in the interface (Schulman & Rideal 1937), arising most probably from hydrogen bonding between the phenolic groups of hexyl resorcinol and the soap molecule (Rideal 1937; Alexander 1942b).

With increase of soap concentration beyond that corresponding to *B* (or *E*), the biological activity falls, ultimately to zero (*BC* in figure 7).

TABLE 1

(0.025 % hexyl resorcinol in all cases. Interfacial tension denoted by I.T. and hexyl resorcinol by H.R.)

	sodium cholate	sodium oleate	C.T.A.B.
critical concentration, soap alone (%)	0.25	0.07	ca. 0.01
critical concentration in presence of H.R. (%)	0.015	ca. 0.05	0.0032
I.T. (dynes/cm.) at minimum, soap alone	9.0	0.6	ca. 4
I.T. (dynes/cm.) at minimum, in presence of H.R.	4.2	0.5	0.3
concentration of soap at maximum acceleration (%)	—	ca. 0.05	0.004
acceleration at maximum (%)	0	300	350

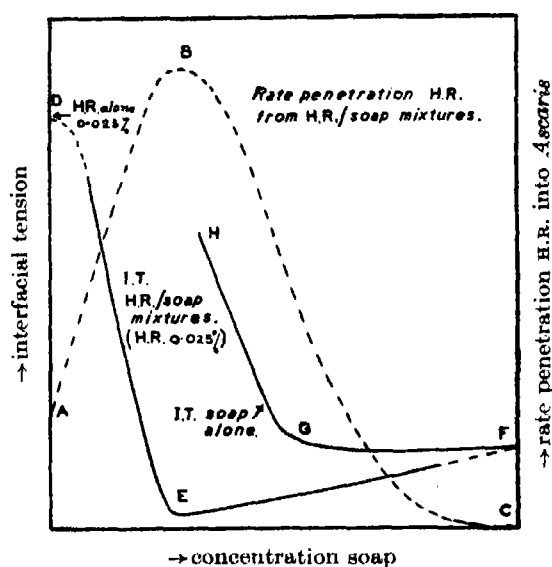


FIGURE 7. 0.025 % hexyl resorcinol/soap mixtures. General diagram to show the effect of soap concentration upon the interfacial tension and upon the biological activity as measured by the rate of penetration of hexyl resorcinol (H.R.) into *Ascaris*.

Over this range of soap concentration the interfacial tension rises (*EF* in figure 7), ultimately reaching a value which approximates to that of the soap alone.

The critical concentration for micelle formation is lowered by the presence of hexyl resorcinol (from *G* to *E* in figure 7), a further indication of association between the interfacial soap and hexyl resorcinol molecules.

The soaps penetrate *Ascaris* relatively slowly, if at all, both alone and when hexyl resorcinol is present under its optimum conditions for penetration.

The above data suggest a simple explanation for the influence of soap upon the rate of penetration of hexyl resorcinol into *Ascaris* and one which is in accord with established physico-chemical principles. It also serves to explain many observations on other biological systems, examples of which are quoted below.

From the observed parallelism between the fall of interfacial tension along *DE* and the increased rate of penetration along *AB* it seems reasonable to conclude that the rate of penetration of hexyl resorcinol from soap/hexyl resorcinol mixtures is intimately related to the interfacial activity of the system. In line with this is the observation that with the different soaps the acceleration at the maximum is in the order of interfacial activity, as pointed out above. Also, with hexyl resorcinol alone, the rate of penetration increases as the interfacial tension decreases, as shown in figure 6.

As the soap concentration is increased further, the micellar region is reached, the interfacial tension then becoming constant and thus setting a limit to the biological activity. Any further increase of soap concentration beyond that corresponding to *B* or *E* only increases the proportion of soap in the micellar form, leaving the concentration of single molecules, which determines the interfacial activity, sensibly constant (Alexander 1942*a*). When hexyl resorcinol is also present in fixed amount it will distribute itself between the micelles and any other interface present (e.g. oil/water or *Ascaris*/water), thus tending to reduce the amount adsorbed at the *Ascaris*/water interface, and consequently tending to diminish the rate of drug penetration. (In addition, the interfacial tension will be raised, since the mixture has a lower value than the soap alone, as shown above, and this also will tend to reduce somewhat the rate of penetration.) By sufficiently increasing the soap concentration, all the hexyl resorcinol can be effectively held by the micelles so that the mixture shows negligible reduction of the interfacial tension below the value for the soap alone (point *F* in figure 7) and also complete prevention of drug penetration (point *C*), since the soaps penetrate *Ascaris* very slowly if at all.

Calculation of the soap effect

It is of great interest to see how far the variation with soap concentration of the rate of penetration of hexyl resorcinol into *Ascaris* can be predicted from calculations based upon physical data only. Two regions have clearly to be considered, namely those before and after micellar aggregation. For reasons given below calculation has to be restricted to the case of C.T.A.B. (see figure 3).

In the region before aggregation any detailed computation appears impossible with our present limited knowledge of the composition of the interfacial film. However, some parallelism between biological and interfacial activities is to be expected, and that part of the curve up to the optimum (corresponding to *AB* in figure 7), was calculated assuming a linear relationship between interfacial tension and biological activity. The optimum rate was taken as 280 % (relative to hexyl resorcinol alone as 100 %), since this value gives the best fit over the micellar region, as shown below.

Turning now to the micellar region, a much more exact approach is possible. Only two assumptions have to be made and both are very plausible.

The first assumption is that the equilibrium for the reaction (C.T.A.B. + hexyl resorcinol)_{in solution} \rightleftharpoons (complex)_{in interface}, lies very largely to the right, i.e. that

the interfacial complex has a very high stability. Work on related systems in monolayers gives definite support for this assumption. In the cases of sodium oleate and sodium cholate the complexes would be of much lower stability since competition arises from unionized fatty acid, and owing to lack of equilibrium constants these systems cannot at present be quantitatively explored.

The calculation is outlined below.

Concentration of hexyl resorcinol = 0.025 %, i.e. solution contains 7.74×10^{17} mol./ml. Concentration of C.T.A.B. = C_s %, i.e. solution contains $C_s \times 1.65 \times 10^{19}$ mol./ml. Critical micellar concentration for C.T.A.B. = 0.004 %. Hence the number of C.T.A.B. mol./ml. free for micelle formation = $(C_s - 0.004) 1.65 \times 10^{19}$. From the above assumption regarding the stability of the complex, one C.T.A.B. molecule *in the micelle* takes up one hexyl resorcinol molecule from solution. Hence the number of hexyl resorcinol mol./ml. left = $7.74 \times 10^{17} - (C_s - 0.004) 1.65 \times 10^{19}$.

The second assumption has now to be invoked; this is that the rate of penetration of hexyl resorcinol is proportional to the concentration of *free* molecules in solution. This is very reasonable since the interfacial tension varies but little in the region where micelles are present. The variation of biological activity, as measured by the initial rate of hexyl resorcinol penetration, with C.T.A.B. concentration can thus be readily calculated, giving the dotted curve shown in figure 3. In this the rate at the optimum has been taken as 280 % to give the best fit for the whole curve, although even if this is taken as 350 % the general shape is not markedly altered.

The general agreement is surprisingly good, the most striking point perhaps being the agreement between the observed and calculated concentrations of C.T.A.B. necessary for complete inhibition of hexyl resorcinol penetration. (It should be realized that this agreement does not depend upon the particular value assumed for the optimum rate of penetration.)

Application to other biological systems

The simple picture outlined above of a competition between micelles and a biological interface should be of general validity not only for all types of phenols but also for many other biologically important compounds when present in aqueous soap solutions. Whether the soap is already present in the system, e.g. as bile salt or salt of fatty acid, or is added intentionally, as for example in many insecticidal and germicidal sprays, should be immaterial. Also, since the effects are due primarily to changes in the medium, a similar behaviour would be expected in other biological systems, subject of course to modification in some instances by specific interactions between chemical groupings on the biological surface and the soap or drug molecules. An examination of the literature does indeed show many phenomena which may be explained in terms of the above concepts.

Billard & Dieulafoy (1904) found that the toxic effect of curare, injected intraperitoneally into guinea-pigs, could be augmented by the addition of low concentrations of soap and decreased by higher concentrations. Frobisher (1927),

examining the germicidal activity of phenol/sodium oleate mixtures against *Bacillus typhosus*, found an optimum soap concentration for a given phenol concentration, and suggested that the inhibition by the higher soap concentrations arose from the soap coating the bacteria with a more or less solid soap film acting as a protective covering. However, more recent studies of surface films of soaps and synthetic detergents show that such 'skins' are not formed under the conditions used by him, and in the present experiments with *Ascaris* their absence has been demonstrated experimentally, as pointed out above.

More recently Petroff, Herman & Palitz (1941), using 1:5000 and 1:10,000 solutions of various synthetic detergents with phenols, observed enhanced activity against *Bacillus pyocyaneus*, a very resistant type of bacteria. It is certain from published data that, save possibly in the case of sodium heptadecyl sulphate, these concentrations are below the critical micellar region, so that the experimentally observed activation is readily explicable. Ordal, Wilson & Borg (1941) examined four fatty-acid soaps and also sodium dodecyl sulphonate, with phenol as the antiseptic, against *Staphylococcus aureus* as test organism. They obtained varying degrees of acceleration of the antibacterial action of the phenol. In an extension of this work Ordal & Deromedi (1943) examined several halogenated phenols in the presence of sodium dodecyl sulphonate (0.02 and 0.1 %) and Aerosol OT (0.02 %) at pH ranging from 6.0 to 9.7. The enhanced activity they observed at pH 6 is readily understood since the detergent concentrations would be below the critical micellar region at the phenol concentrations used. The loss of activity on increasing the pH to 9.7 can be ascribed to ionization of the phenol and hence reduced surface activity of the mixture.

There are many recorded examples of inhibition of biological activity by soaps in addition to those mentioned above. In the case of soap/phenol systems, see for example Tilley & Schaffer (1925, 1930) and Hampil (1928). The soap concentrations used were usually very much higher than the critical micellar concentration, so that the detoxifying effect of the soap can reasonably be ascribed to the same mechanism as suggested in this paper for hexyl resorcinol inhibition under similar high soap concentrations.

Closely related to the topics under discussion is the recent observation by Valko & Dubois (1944), that detoxication of surface active cations on bacteria can be brought about by addition of surface active anions. It is well known that this causes a precipitation and reduced surface or interfacial activity, this principle being the basis of one suggested method for analysis of synthetic detergents (Preston 1944). If, as the work described in this paper suggests, there is some correlation between interfacial and biological activity, then the reason for this detoxication is clear.

The question of the applicability of the above principles to bacterial systems is being investigated by Dr A. J. H. Tomlinson of Emergency Public Health Laboratory Service, Cambridge, using mixtures of synthetic detergents with various phenols, and his preliminary experiments have given results in accord with those predicted from interfacial tension measurements.

In addition to their obvious relevance to those types of insecticidal and germicidal sprays which contain soaps or wetting agents, the principles developed here may also play a part in the mechanism of fat absorption, a much debated topic which has been further studied recently by Frazer and his collaborators (Frazer 1943; Frazer, Schulman & Stewart 1944).

The penetration of hexyl resorcinol through the cuticle of Ascaris

The results recorded in this paper throw some light upon the mechanism of the transport of this drug through the *Ascaris* cuticle, and may also be pertinent to its action on other organisms.

Under the experimental conditions employed here it can reasonably be assumed that the rate of penetration into or through the cuticle is the limiting factor in the passage of hexyl resorcinol into *Ascaris*. Of the various mechanisms by which penetration might occur two call for particular discussion: first, by solution in the cuticle followed by an ordinary diffusion process, the driving force arising from the concentration gradient across the cuticle; secondly, by a two-dimensional interfacial spreading along lateral sub-microscopic pores or canals, the driving force being the interfacial spreading pressure. The possible biological significance of this latter mechanism was suggested by Rideal (1938), and recently elaborated by Hurst (1943) to explain certain aspects of penetration of insecticides.

On the latter explanation the acceleration produced by soaps (in the range before micellar aggregation) would be ascribed to the increased spreading pressure of the mixture; on the former to the increased interfacial activity of the soap/hexyl resorcinol complex augmenting the amount of drug on the *Ascaris* surface. This increased amount may arise either from a true increase per unit area (since we are not dealing with equilibrium conditions, but with a rate process), or from an opening-up of the outer layers of the cuticle, which by presenting a greater area for adsorption increases the apparent rate of drug penetration.

The observed parallelism between degree of acceleration and interfacial activity is clearly equally well explicable on either mechanism, but if the latter is correct some correlation between the penetration rates of hexyl resorcinol and of soap would be expected. It has been demonstrated above in the case of *Ascaris* that the amounts and rates of soap penetration are only relatively small, and are unaffected by the simultaneous penetration of hexyl resorcinol under its optimum conditions. Hence, in this case, penetration by a two-dimensional interfacial spreading cannot be of importance.

The marked difference between the penetration rates of ionized substances such as the soaps, and of unionized compounds such as hexyl resorcinol, is further shown by the effect of pH on the penetration of nicotine, where the rate becomes a minimum upon ionization (Trim, unpublished). Also the cuticle is clearly impermeable to colloidal particles, since even soap micelles with diameters of the order of 50 Å (i.e. at the lower limit of the colloidal range), are unable to penetrate.

It is hoped, however, to examine in considerably more detail the mechanism of hexyl resorcinol penetration through *Ascaris* cuticle, and to carry out parallel experiments upon synthetic membranes.

CONCLUSION

Although all the biological data referred to in this paper have been obtained from *in vitro* experiments, yet the understanding of the basic phenomena involved should be of considerable value in any attempt to increase the *in vivo* efficiency of hexyl resorcinol and other anthelmintics. Some preliminary experiments (Trim 1944) upon the effect of such substances as intestinal mucin which are normally present in the host intestine have shown the importance of further investigations in order to define the conditions for optimum *in vivo* efficiency of hexyl resorcinol.

One further point of much interest arising from this work is in connexion with drug efficiency in general. For a given type of drug the biological activity often passes through a maximum as the non-polar portion of the molecule is increased; for example, optimum activity is reported at the hexyl member in the alkyl resorcinols (e.g. Ferguson 1939). In this, as in many other examples, the maximum appears to arise from solubility limitations, which thereby set a definite limit to the rate of penetration into the biological system. By the use of suitable concentrations of soaps it may now be possible to overcome such difficulties and thus to enhance the biological activity of the higher members.

Finally, by controlling the relative amounts of drug and soap, and by the use of soaps of varying surface activity, it may be quite feasible to control, not only the intensity of biological activity but also the duration, at any desired level up to the optimum. With drugs which have undesirable effects on the host, this clearly is of great value.

We should like to express our indebtedness to Professor E. K. Rideal, F.R.S., to Professor A. C. Chibnall, F.R.S., to Professor D. Keilin, F.R.S., and to Dr E. Friedmann for their interest in this problem. This work forms part of a programme of investigations into the action of anthelmintics being carried out for the Agricultural Research Council by one of us (A.R.T.).

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Siderocytes in mammalian blood

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Since a review of the conditions under which siderocytes appear will support a hypothesis that it is an ageing erythrocyte at least as strongly as Grüneberg's (1941*a*) theory that it should be considered as a young cell, a search was made in blood films of stored mammalian blood and large numbers of siderocytes were found.

The conditions affecting the rate of appearance of the siderocytes were studied, and it was found that adverse conditions would hasten their appearance.

The relationship of the siderotic material to the 'easily split' blood iron was also considered, and it seems probable that both are derived from a special and identical fraction of 'haemoglobin', and that this phenomenon is related to an intracorpuseular bile pigment formation.

The occurrence of siderocytosis after the ingestion of acetyl phenylhydrazine by a 'normal' human being was followed, and a close correlation between siderocytosis, erythrocyte destruction and urinary siderosis as described by Peyton Rous (1918) is shown.

The siderocyte extrudes its siderotic granules and reverts to a state at present morphologically indistinguishable from the normal erythrocyte, but appears to be susceptible of phagocytosis at this stage.

The application of siderocyte counts to clinical medicine is suggested.

INTRODUCTION

A siderocyte is an erythrocyte which contains granules which can be stained by the Prussian blue reaction. These cells were so named by Grüneberg (1941*a, b*), who found them in large numbers in *Mus musculus* L. showing flex-tail anaemia. He was not entirely accurate, however, in stating that such erythrocytes were unknown. Stainable iron in red cells had been reported at the end of the last century (Macallum, 1895; Erlich & Lazarus 1898) and also in 1928 (Proescher & Arkush). Doniach, Grüneberg & Pearson (1943) reported siderocytes in adult human blood in one case of biliary cirrhosis with splenectomy, in four cases following splenectomy and in two cases of chronic uraemia.

Grüneberg had concluded that the siderocyte was a young erythrocyte, also that the iron was anabolic, and probably not associated with the 'easily split' blood iron (E.S.I.) studied by Lemberg & Legge (1939), Legge & Lemberg (1941) and by Barkan (1927).

He (Grüneberg 1941*a*) had also noted that the livers of the anaemic mice contained considerable deposits of stainable iron (haemosiderin).

A consideration of the conditions in which the siderocyte had been found, ignoring for a moment the genetical anaemia of the mouse, the aetiology of which is at least obscure, would, *a priori*, suggest that the siderocyte was an ageing erythrocyte. If this were to be true, it becomes probable that the iron is catabolic, and thus might provide a source for the E.S.I., and also for the hepatic siderosis of Grüneberg's mice.

Grüneberg, however, had produced indisputable evidence that the siderocytes in the flex-tailed mice were confined to the intermediate cell generation, and were within a few hours of their liberation into the circulation. Nevertheless, it is possible for a cell to be near to both ends of its life cycle at a given moment.

Barkan (1927) and Lemberg & Legge (1939) had shown that when the E.S.I. was produced, an intracorpuseular bile pigment formation took place, and later Lemberg & Legge (1943) produced intracorpuseular choleglobin and biliverdin in rabbits by the use of acetyl phenylhydrazine.

Rous (1918) described siderotic granules in the urine of patients suffering from either pernicious anaemia or haemochromatosis, and suggested that these might be used as a diagnostic method.

Since these studies were well under way, and between the writing of the preliminary results (Case 1943) and their publication, Granick (1943) has reported the occurrence of non-haematin iron in erythrocytes in the teased spleens of the horse, man, guinea-pig and rabbit, and suggested that such cells were degenerating erythrocytes, and that the degeneration was produced by a secretion of cells lining the splenic sinusoids. Whilst these conclusions are in general true, the methods used by Granick are inadmissible, as the hydrogen sulphide reagent will produce siderocytic change in normal unfixed erythrocytes.

The following work, largely on stored blood, has been carried out in an attempt to elucidate the physiology of the siderocyte, and the results appear to link up with these apparently unconnected observations of other workers.

METHODS AND MATERIALS

Staining technique. Thin blood films, made on glass slides, were air dried and fixed in methyl alcohol. They were then stained as follows: placed in strong yellow ammonium sulphide solution for 2 hr. Rinsed in tap water. Placed in a freshly prepared mixture of 2% potassium ferricyanide and 2% hydrochloric acid for 20 min. Rinsed. Differentiated for 30 sec. in 1% hydrochloric acid in 95% alcohol. Rinsed in slightly alkaline water (Birmingham tap water). Counter-stained with 1% eosin or 1% Biebrich scarlet solution.

Since these preliminary studies were completed, a better staining technique, using $\alpha\alpha'$ -dipyridyl and potassium thiocyanate in dilute hydrochloric acid has been elaborated and described (Case 1944) and has now entirely superseded the ammonium sulphide technique.

Quantitatively, however, the results are not influenced by this change of technique.

Counting. 1000 red cells were counted in each case, and the number of siderocytes noted. Only cells containing definitely blue-green granules were counted as siderocytes.

Haemoglobin estimations. These were carried out by the alkaline haematin technique of Clegg & King (1942) using a 'Spekker' photoelectric absorptiometer and green filters. The standard error of estimate was $\pm 1.0\%$.

Bile pigment estimations. No attempt to investigate the nature of the bile pigment has been made in these investigations, but the total diazotizable pigment was estimated as bilirubin, using the method of King, Haslewood & Delory (1937). The standards were made from pure bilirubin, and the 'Spekker' instrument was again used.

Non-haemoglobin iron estimations. These were carried out by Dr Gaddie, of the General Hospital, Birmingham, using the $\alpha\alpha'$ -dipyridyl method of Thorp (1941).

Hanging-drop preparations were made in the following way. A drop of blood suitably diluted with isotonic saline containing a trace of acetyl phenylhydrazine was placed on a cover-slip with a drop of ferricyanide reagent adjacent to it. The cover-slip was inverted over a well in a slide, and the edges sealed with vaseline.

Clot-tubes were prepared by drawing out glass tubes to form a connected series of bulbs; these were filled with blood which was allowed to clot, the drawn-out portions broken and the ends sealed with paraffin wax.

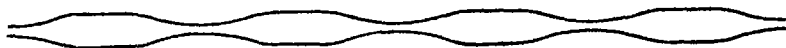


FIGURE 1

Urine examinations were made by centrifuging urine for 40 min. The tube was emptied and the drop remaining on the bottom was placed on a slide which was coated with a thin layer of dried human plasma. The drop was evaporated in an oven at 60° C, fixed with methyl alcohol, and stained in the same way as blood films. Only an approximate estimate of urinary siderosis was formed.

Human blood was obtained from donors in the blood donor service, and taken by the usual operators into either 3.8 % sodium citrate solution, using 100 ml. citrate and 440 ml. blood, or else 430 ml. of blood were taken into 100 ml. 3.0 % sodium citrate and 10 ml. 30 % glucose (M.R.C. glucose citrate).

Cat and dog blood was obtained from nembutal anaesthetized animals which were being submitted to other operative procedures.

The human subject (R.A.M.C.) was a healthy adult male, aged 28.

EXPERIMENTS

(1) *The appearance of siderocytes in stored blood*

Blood, taken into 3.8 % sodium citrate, was divided into two parts. Part 1 was inverted at every sampling, so that it was reoxygenated as well as mixed; part 2 was subdivided into 20 equal parts in sterile test-tubes, and each sample discarded after making the film. Slides were made daily for 19 days, the bloods being stored at 20° C. In addition, frequent non-haemoglobin plasma iron and bile pigment estimations were made on the oxygenated sample. The results are shown graphically in figure 2.

It will be seen that siderocytes appear in both bloods after 24 hr., but that in the oxygenated blood the graph has an irregular cyclical tendency. The plasma iron and bile pigment increase in a parallel manner, and the amounts formed are compatible with the idea that they are formed from haemoglobin.

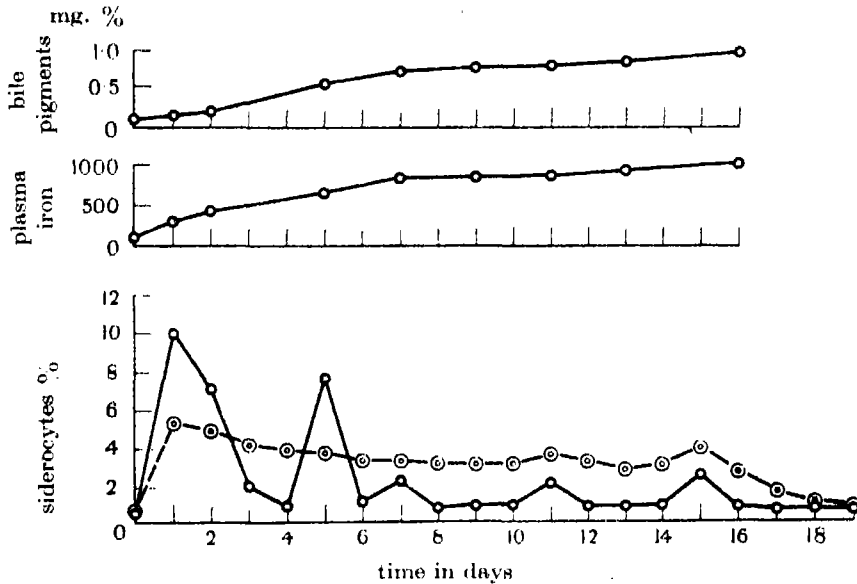


FIGURE 2. ○—○ inverted. ⊙—⊙ unshaken.

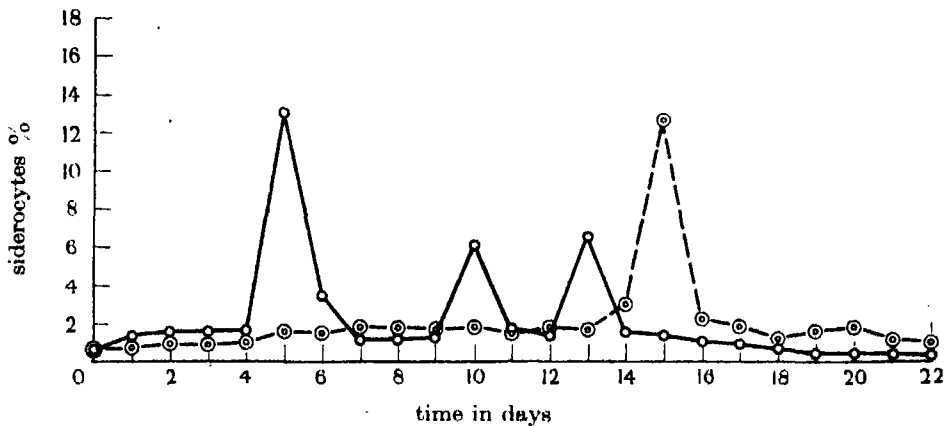


FIGURE 3. ○—○ 3.8 % NaCit. ⊙—⊙ MRC cit. glucose.

The total erythrocyte count had not materially altered during the experiment, being 4.15×10^6 on the first day and 4.20×10^6 on the nineteenth day.

After the second day, iron granules, morphologically similar to the intracellular bodies, appeared free in the plasma, and the appearances suggested that they had

been extruded from the erythrocytes. Occasionally leucocytes were seen containing iron granules, and as such intracellular granules had not been seen initially, it was concluded that the leucocytes had phagocytosed them.

Two more blood samples were examined, one taken into 3.8% sodium citrate, and one into M.R.C. glucose-citrate. These were stored in a refrigerator at 4° C and shaken daily, films being made from blood withdrawn by a sterile needle and syringe through a rubber diaphragm in the stopper of the bottle. The results are shown in figure 3. Again the irregular cyclicality is seen, but the first siderocyte peak appears at the fifth day in the blood with no carbohydrate and at the fifteenth day in the glucose-preserved blood.

(2) *The effect of temperature*

150 ml. of cat's blood were withdrawn into 10 ml. of 3.8% sodium citrate. This citrated blood was divided into three parts, one being stored at 4° C, one at 20° C and one at 37° C. Films were made hourly for 6 hr., then at longer intervals. The results are shown in figure 4. It is shown that siderocytes appear in cat's blood, and that within the limits stated cold inhibits the change and heat accelerates it.

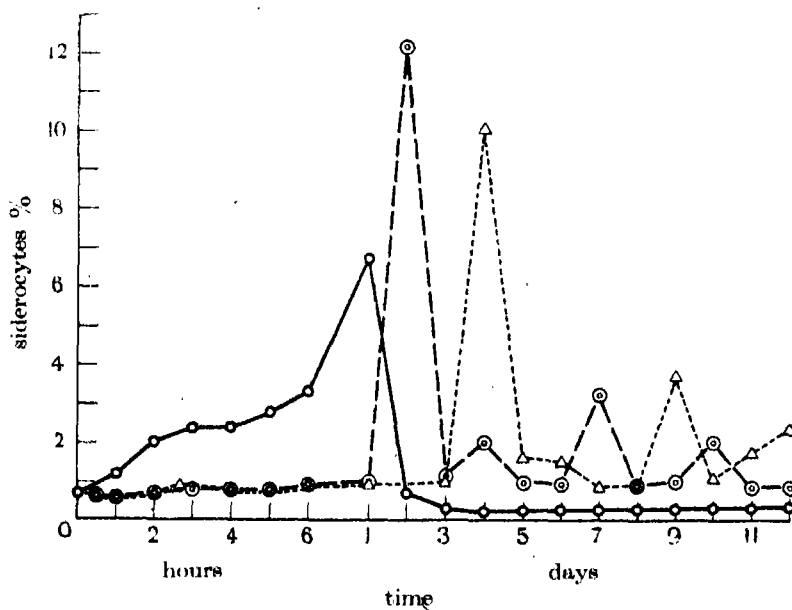


FIGURE 4. ○——○ 37° C. ⊙——⊙ 20° C. △-----△ 4° C.

(3) *The effect of oxygenation*

50 ml. of dog's blood were withdrawn into 10 ml. of 3.8% sodium citrate, and divided into three parts. One part was saturated with carbon monoxide and stored in a tube filled with this gas, and sealed with a rubber cap; one part was stored under

a 2 in. layer of sterile liquid paraffin, and one part in air. The bloods were stored at 20° C, and films made daily. It will be seen that the blood kept from contact with the air shows an early and well-marked siderocyte response, and that carbon monoxide produces an apparent inhibition of this change.

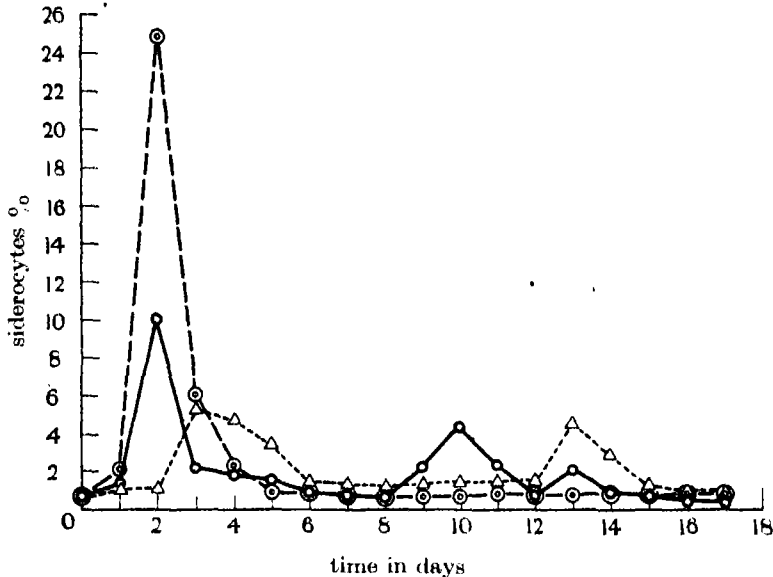


FIGURE 5. ○—○ air. ⊙—⊙ under oil. △—△ CO.

(4) *The effect of the anticoagulant*

Some of these experiments were repeated with heparinized blood, oxalated blood, and defibrinated blood. Results essentially similar to those in blood diluted with sodium citrate were obtained.

Clots were prepared in the special tubes as follows:

- (1) Fresh whole human blood was drawn up into three tubes and allowed to clot.
- (2) Blood from the same donor was taken into 3.8% sodium citrate, placed in sterile centrifuge tubes and spun until the supernatant plasma was clear. This plasma was pipetted off into a sterile vessel. The surface layer containing an excess of leucocytes was removed from each tube with a capillary pipette and collected into one tube.
- (3) The remaining red cells were then washed with three changes of Ringer-Locke fluid, and each time the top layer was removed and discarded. It was assumed that the remaining red cells were free from leucocytes, as none could be detected in a blood film after lengthy search. Both these cells and the cells with an excess of leucocytes were mixed with portions of the plasma to give the original haematocrit reading, the calculated amount of calcium chloride added, the bloods drawn up into the special tubes and allowed to clot. All the tubes were broken into lengths and the ends

sealed. Some of each series were stored at 4, 20 and 37° C respectively. Bulbs were broken open daily, the clot fixed in methyl alcohol, and paraffin sections made. These were stained by the method described.

The tightness of packing of the red cells precluded accurate counting, but the siderotic granules appeared at apparently equal rates in each series of tubes, the rate being influenced by temperature as in the previous experiments.

Thus it appears that whilst the type of anticoagulant may influence the rate of appearance of the siderocytes, it does not determine their formation. The presence of leucocytes has no apparent influence on the phenomenon.

(5) *The action of chemical agents*

Blood was treated with a variety of chemical agents, chosen largely at random, with the exception of acetyl phenylhydrazine, which was selected because of its property of lowering the erythrocyte count in animals.

The results of the phenylhydrazine experiments are shown in table 1, and essentially similar results were obtained using 400 µg. % of potassium dichromate, silver nitrate, or lead nitrate, or 0.4 ml. % of carbon tetrachloride or carbon disulphide.

TABLE 1. 100 ML. SAMPLES OF BLOOD IN SODIUM CITRATE DILUENT, TREATED WITH ACETYL PHENYLHYDRAZINE FOR 3 HR. SIDEROCYTE COUNTS PERFORMED AT THE END OF 2 HR. ACETYL PHENYLHYDRAZINE ADDED AS A SOLUTION IN 1 ML. OF ISOTONIC SODIUM CHLORIDE

agent used	Hb g. %	Hb drop mg. %	% Hb drop	plasma Fe µg. %	% Hb equiva- lent of Fe rise	bile pigment mg. %	sid. %
250 µg. acetyl phenylhydrazine	10.26	540	5.0	2000	5.1	0.5	20.0
500 µg. acetyl phenylhydrazine	10.20	600	5.6	2200	5.4	0.55	18.2
1000 µg. acetyl phenylhydrazine	10.25	550	5.1	2100	5.25	0.45	21.0
1 ml. isotonic sodium chloride	10.80	0.0	0.0	150	—	0.1	2.3

It is seen that a siderocytosis is produced in a relatively short time, and that this phenomenon is accompanied by a fall in the total blood pigment, amounting to between 4 and 7 % of the total value, and a corresponding rise in non-haematin plasma iron. There is also a rise in bile pigment, but phenylhydrazine and most of the other agents used destroy bilirubin *in vitro*.

It also seems, from the results with acetyl phenylhydrazine, that a limit is reached after which no further breakdown of haemoglobin takes place.

The siderocyte curve of human blood in 3.8 % sodium citrate was next followed. 500 µg. % of phenylhydrazine were added, and hourly siderocyte counts and

haemoglobin estimations were performed. The results are shown in figure 6. A second addition of acetyl phenylhydrazine was made after 12 hr.

The curve shows that there is a rapid siderocyte production; the granules then disappear, and no second wave is produced by the addition of further reagent. There is a haemoglobin drop at the time of the siderocyte peak, about 6% of the total pigment being degraded.

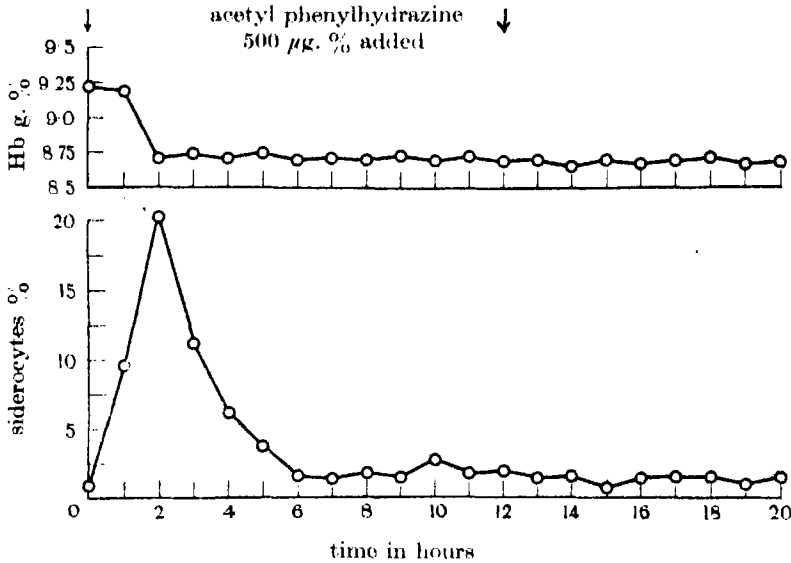


FIGURE 6

(6) *The effect of the cell membrane*

Blood, taken into 3.8% sodium citrate, was laked by the addition of an equal quantity of distilled water and a trace of white saponin. The laked blood was placed in a cylinder and rotated until a clear solution was obtained. A drop of this fluid was

TABLE 2. BLOOD, IN 3.8% SODIUM CITRATE, HAEMOLYSED WITH DISTILLED WATER AND WHITE SAPONIN. A, FILTERED TO REMOVE STROMATA. B, UNFILTERED

concentration of acetyl phenylhydrazine µg. %	A			B		
	Hb g. % at 0, 3 and 18 hr.			Hb g. % at 0, 3 and 18 hr.		
	0	3	18	0	3	18
0	7.31*	7.32	7.30	7.71*	7.72	7.70
50	7.32	7.31	7.33	7.71	7.70	7.72
250	7.30	7.30	7.29	7.71	7.70	7.70
500	7.31	7.32	7.33	7.71	7.72	7.70
1000	7.30	7.33	7.29	7.70	7.71	7.69

* The difference in the initial haemoglobin levels is probably due to adsorption of pigment by the filter bed.

placed in saturated sodium chloride solution, and examined with a microscope. When complete haemolysis was achieved, no cells were visible (Woodhouse & Pickworth 1930). The laked blood was divided into two parts, and one part was filtered through a Seitz K filter to remove stromata. Both aliquots were treated with various concentrations of acetyl phenylhydrazine, and haemoglobin estimations performed after 3 hr. and after 18 hr. The results are shown in table 2. It will be seen that as no haemoglobin degradation was produced in either specimen, the integrity of the erythrocyte appears to play a part in the phenomenon of haemoglobin breakdown.

(7) *Extrusion of the granules*

Hanging-drop preparations were made in duplicate by the method described. One slide was immediately tilted to mix the staining reagent and the cells, and examined with the microscope. The extracellular fluid was free from formed structures, and the cells were relatively free from granules. The other slide was examined without mixing, and after about 1 hr. small refractile bodies, apparently linked together, appeared centrally in the cells, migrated to the periphery, and in many cases could be seen to pass through the cell membrane and float free in the extracellular fluid. These granular structures then appeared as three or more granules apparently linked together. At this point the slide was tilted, and the staining reagent mixed with the drop of blood-mixture. It was now possible to see that the extracellular bodies gave a positive iron reaction, as did many of the granules in the cells.

This experiment was repeated using 1% hydrogen sulphide solution in normal saline instead of acetyl phenylhydrazine, and omitting the iron reagent. Again granules formed in the cells, and became dark. Some of these granules were extruded.

(8) *The susceptibility of the siderocyte to phagocytosis*

Blood was taken into 3.8% sodium citrate, and divided into three parts.

One part was at once centrifuged and the buffy coat from about 200 ml. removed with a capillary pipette. This buffy coat was suspended in a little of the supernatant plasma and placed in an incubator at 37° C.

One part of the blood was stored in blood-bank conditions at 4° C, and the remaining part was treated with 500 µg. % acetyl phenylhydrazine and allowed to stand at 20° C for 3 hr.

At the end of this time a part of the blood which had been stored at 4° C was treated with 500 µg. % of acetyl phenylhydrazine. The buffy coat suspension was divided into three parts and the following preparations made:

- (1) 1 ml. of buffy coat and 1 ml. of blood which had been kept at 4° C were mixed.
- (2) A similar preparation was made of the blood stored at 4° C but treated with phenylhydrazine immediately before mixing.
- (3) A similar preparation using the blood treated at 20° C for 3 hr. was made.

Aseptic precautions had been observed throughout all these steps.

These preparations were returned to the incubator for 1 hr., and shaken at 5 min. intervals. Films were then made from them stained for iron, and counterstained with a buffered Romanowsky stain (Hynes 1942).

An examination of the films revealed that in preparation (1) the leucocytes remained indifferent to the erythrocytes; in preparation (2) many siderocytes were seen, and many polymorphonuclear leucocytes had ingested iron granules; in preparation (3) fewer siderocytes were seen, but much extracellular iron was visible. Many polymorphs had ingested iron granules, and some of the leucocytes, mainly mononuclears, had ingested erythrocytes. The erythrocytes appeared to be disintegrating into a granular mass of debris within the leucocytes, but only some of the ingested cells showed siderotic granules.

This experiment suggests that an erythrocyte becomes susceptible to phagocytosis at about the time that it becomes a siderocyte, or after the granules are extruded.

(9) *The effect of phenylhydrazine in vivo, and the relationship of siderocytosis to urinary siderosis*

Acetyl phenylhydrazine is used therapeutically to reduce the erythrocyte count in polycythaemia rubra.

In view of the foregoing results it seemed possible that such a reduction would be preceded by a siderocytosis, and as the extruded granules offer an obvious source for the urinary granules of Rous, it seemed possible that the latter might also appear.

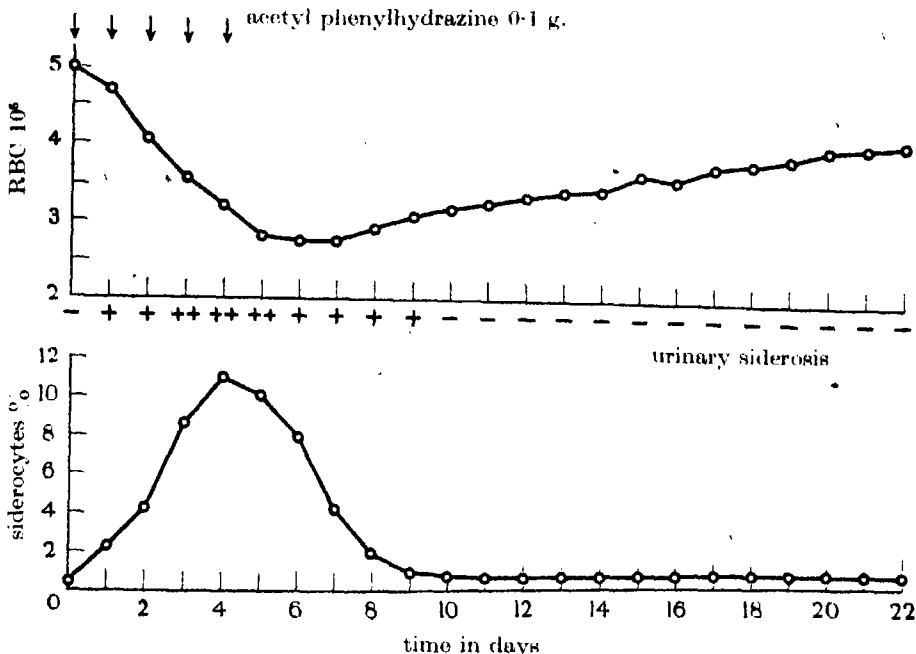


FIGURE 7

The occurrence of high circulating siderocyte levels has been observed in untreated pernicious anaemia and in haemochromatosis, but this will be discussed in another paper.

0.1 g. of acetyl phenylhydrazine was administered daily for 4 days to a normal human subject. The blood count, siderocyte count, haemoglobin, and urinary siderosis examinations were made daily for 22 days. The results are shown in figure 7. For the sake of clarity, the haemoglobin values are not plotted, but they followed the blood count exactly, a normochromic anaemia resulting. It is seen that the fall in blood count is accompanied by a marked siderocytosis, and that during the recovery period, when young cells might be expected in the circulation, the siderocyte count falls to normal levels.

The urinary siderosis is obviously closely correlated with the siderocytosis.

DISCUSSION

The foregoing results show that siderocytes appear in the blood of the cat, dog and of man when it is stored or treated with certain chemical agents, some of which are known to cause anaemia *in vivo* (phenylhydrazine, salts of lead).

Apparently all the red cells go through a stage as a siderocyte, but the siderotic granules are extruded, and the histological methods used afforded no morphological differentiation of cells in a pre- and post-siderotic phase. Thus the plotted curves of siderocyte levels become the expression of two factors, a wave of siderocyte production and a wave of siderocyte disappearance which continuously modifies the first curve. Thus at no stage is a level of 100% siderocyte count approached.

The oxygenation of the cell also plays a part, both in modifying the rate of siderocyte production and in altering the cyclicity of the change (figure 2).

The possibility that the change is reversible must be considered, and these studies yield no direct evidence on this point, though the steadily rising plasma iron and bile pigment curves are against such a hypothesis; it is difficult to conceive of such a reversal being possible after extrusion has occurred.

The experiments with blood stored under liquid paraffin show that a more complete exclusion of air accelerates the change, if it may be assumed that liquid paraffin is inert, which is possibly an unwarranted assumption. Saturation with carbon monoxide produces an apparent inhibition of the change. Legge & Lemberg (1941) showed that carbon monoxide would produce an apparent inhibition in the splitting off of the E.S.I.

Storing blood at different temperatures showed that cold would inhibit the change, and that heat would accelerate it, within the limits chosen (4–37° C).

Whilst it has been shown that the change will occur in clot, defibrinated blood, and bloods rendered incoagulable with a variety of substances, and that therefore the phenomenon is not determined by the anticoagulant, it is also shown that the composition of the anticoagulant may modify the rate of change. Thus in figure 3 it is seen that the first significant wave of siderocyte production takes place on the

fifth day in blood taken into sodium citrate, whilst the addition of glucose to the diluent retards this process to the fifteenth day.

These results are highly significant in the light of the work of Mollison & Young (1942), who found that donor erythrocytes stored in citrate only would not survive on transfusion if the blood had been stored for more than 5 or 6 days, but that blood stored in citrate containing carbohydrate (glucose in many of their experiments) was as good as fresh blood until about the fourteenth day.

These figures are in accord with the theory that the siderocyte is an effete cell which is susceptible of phagocytosis, and will thus be removed from the circulation.

This concept is also borne out by the results of the buffy suspension experiments.

The clot experiments also show that the presence of phagocytic cells is not essential to the production of granules of stainable iron. The experiments of Muir & Niven (1935) had suggested that such cells were an essential in the degradation of haem pigment.

However, Barkan and Lemberg & Legge had shown that both iron and bile pigment could be formed from whole blood, and that the bile pigment was produced by an intracorpuseular mechanism. It is apposite to mention that acetyl phenylhydrazine was one of the agents used by them to demonstrate this *in vivo* (Lemberg & Legge 1943).

The experiments in figure 1 show that concomitantly with the siderocyte production there is a rise in plasma iron and bile pigment, and figure 6 shows that the siderocytosis is accompanied by a diminution of total pigment. These amounts vary slightly from blood to blood, but the total loss of pigment lies between 3 and 6 % of the whole. The increases in iron and bile pigment are of a similar order of magnitude, though the methods used for estimation of bile pigment are not sufficiently accurate to allow the results to be expressed as molecular equivalents.

The experiments with haemolysed blood suggest that the presence of an intact cell membrane plays a large part in the degradation process, though Barkan (1927) showed that the E.S.I. could be split off from laked blood. However, the agents used by him were of a somewhat more drastic character.

The results in figure 6 also suggest that the siderocytic change can only take place once in any given cell, and that only a definite fraction of the blood pigment can be degraded by this particular mechanism.

All these results lead to the conclusion that the siderotic granules are formed from a special fraction of the blood pigment, probably the same fraction which yields the E.S.I.

The hanging-drop preparations confirm the extrusion hypothesis, and the fact that hydrogen sulphide solution can be used as an agent to produce siderocytosis in these experiments leads to the statement made in the introduction, that Granick's (1943) demonstration of what are certainly siderocytes in the spleen cannot be admitted on the grounds stated.

The experiment with acetyl phenylhydrazine *in vivo* suggests that the hypothesis of the siderocyte as an effete cell is probably correct, and also supports the idea that

this cell may be the source of the urinary granules of Rous (1918). The experiment also indicates that siderocyte counts might be useful clinically in assessing haemolytic processes.

CONCLUSIONS

1. The siderocyte appears in large numbers in stored blood and in blood that has been treated with certain chemical agents. Phenylhydrazine is such an agent.
2. Apparently all erythrocytes in these experiments go through a stage as a siderocyte, but the granules are extruded, leaving a cell which is at present morphologically indistinguishable from a pre-siderocyte erythrocyte.
3. As siderocytes have now been demonstrated in mouse, rat and man (Grüneberg 1941*a, b*); in guinea-pig, horse and rabbit (Granick 1943) and in cat and dog (Case 1943), the cell is probably to be found in all mammals.
4. After an erythrocyte has become a siderocyte it becomes susceptible of phagocytosis, and is removed from the circulation in the intact animal.
5. The stainable non-haematin iron is derived from a special fraction of the haemoglobin, and the other side of the breakdown is bile pigment. Therefore, the iron should be regarded as catabolic, and closely associated with, if not identical with, the E.S.I. described by other workers.
6. The siderocyte is probably the source of the urinary siderotic granules described by Rous (1918).
7. The siderocyte probably has importance in clinical medicine.

I should like to express my gratitude to the many people who have helped me with this work, especially to Professor Lancelot Hogben, F.R.S., and Professor J. B. S. Haldane, F.R.S., for encouragement and advice, to Professors G. Haswell Wilson and H. P. Gilding, to Dr M. E. Nutt for assistance with blood counting, and most particularly to Mr C. Lees, of The General Hospital, Birmingham, for his invaluable assistance in preparing material. Part of the expense of this work was defrayed by a grant from The University of Birmingham.

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The reproduction of the house-mouse (*Mus musculus*) living in different environments

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This paper is based upon the study of 8207 wild mice (*Mus musculus*). The bulk of the animals were obtained from 8 January 1942 to 7 January 1943, when 1067 mice were obtained from urban habitats, 1150 from flour buffer depots, 1218 from cold stores and 2033 from corn-ricks, totalling 5468.

Trapping with break-back traps provides a biased sample of the population, larger animals being caught more readily than smaller ones. In collecting from a rick, methods can be employed which yield a virtually complete population.

In the absence of information regarding age, weight is adopted as the basis of classification.

The criterion of fecundity adopted for females is the presence of corpora lutea in the ovary and for males the presence of numerous sperms in the cauda epididymidis. 25% alcohol or methylated alcohol sufficiently preserved whole animals waiting for examination in hot weather, without making the recognition of sperm difficult, or effecting any significant alteration in weight when dried.

The following information has been obtained for mice from the four habitats: (a) They breed throughout the year. (b) The evidence is against seasonal differences either in the percentage number of adult females pregnant, or in the number of embryos per litter.* (c) The number of nestlings per litter found in the ricks varied from 2 to 13, with an average of 5.83. Communal nests are quite common. (d) The sex ratio for urban is 51.83% male, flour depots 50.43%, ricks 44.61% and cold stores 48.19%. (e) Sex is correlated with weight,* i.e. there is a preponderance of males in the lighter weight groups, and of females in the heavier weight groups. (f) Fecundity in the female is reached in the 7.5 g. weight group in all four habitats, though the percentage number reaching it in the ricks and particularly in the cold stores is much lower than in the domestic and flour depot samples.* In the male fecundity is attained at a somewhat heavier weight. (g) The pregnancy rates increase with the weight of the mouse in all four habitats. (h) The proportion of fecund females pregnant during the year is 0.2194 for urban, 0.3166 for flour depots, 0.4060 for ricks, 0.2653 for cold stores. (i) The annual litter productivity is as follows: urban 5.52, flour depots 7.97, ricks 10.22, cold stores 6.68 litters per annum. (j) The average number of embryos per pregnant female is not significantly different between urban, flour depot and rick samples, giving an average of 5.60;* that for the cold stores is significantly higher, namely, 6.37.* There is no correlation with weight of the parent.* (k) On an average a larger number of embryos were

* Statistically significant.

present in the right than in the left horn of the uterus.* (l) The embryo productivity rates for the four environments are as follows: urban 30.91, flour depots 44.63, ricks 57.23 and cold stores 42.55 embryos per pregnant female per annum. The annual daughter productivity rates are: urban 16.22, flour depots 24.79, ricks 31.51 and cold stores 23.15. (m) It is estimated that the nestling production rate is in the order of 3 % less than the embryo rate.

The most striking differences presented by the cold-store mice are their greater weight and somewhat larger number of embryos. The rick mice show the highest productivity rates. The urban rates are lowest. The rates for the mice from the flour depots and cold stores are average. From one depot in particular, the mice had very thin skin and pelage, and torn and crumpled ears.

Special attention may be drawn to the mice in cold storages. They apparently live normally in an environment which has a temperature never more than 15° F and can breed in this cold environment all the year round.

A brief account of a rick mouse population is given.

1. INTRODUCTION

This investigation has involved the examination of 8207 mouse bodies obtained from several different habitats, between 8 January 1942 and 22 July 1943. It was carried out in the Department of Zoology and Comparative Anatomy of the University of Oxford in close association with the Bureau of Animal Population, and was undertaken for the purpose of acquiring knowledge regarding the structure and dynamics of wild mouse populations.

Mus musculus L. is of world wide distribution. According to Schwarz & Schwarz (1943) the British mainland house-mouse is the subspecies *M. musculus domesticus* Ratty. In the warmer climes such as Australia, U.S.A., and south Russia *M. musculus* lives partly in the fields. Farther north in Russia it is largely confined to dwellings. On the mainland of Great Britain too this is largely the case, where it occurs in houses in town and country and in farm buildings and ricks, wherever there is food and a certain amount of warmth and shelter. Paradoxically, it is also found in cold stores. At intervals it increases to such large numbers that it constitutes a serious pest. It exhibits, as do other animals, fluctuations in numbers, which increase at times to the proportions of a plague. Elton (1942) gives a résumé of some of these occurrences. Some indication of the severity of these plagues is given by the following examples. In the Australian plagues of 1916-17, £1,000,000 worth of damage was done to stacks of wheat. Hall estimated a density of seventeen mice per square yard, or over 80,000 to an acre, during migrations, at the centre of the outbreak which occurred in Kern county, California, in 1926. As yet no clear evidence has been brought forward for any rhythm in the fluctuation of their population numbers.

The house-mouse has been recognized from early times in Great Britain as a pest. Thus in the year 930 (Cahalane 1943) cats were included in a national plan to keep down a plague of rats and mice in Wales. The Welsh chief, Howel Dda, standardized the price of cats according to their ability at mouse catching.

The reproduction of wild populations of *M. musculus* has received little attention, but intensive research has been carried out by various workers on the reproductive processes in laboratory stocks. A useful summary is given in *Biology of the laboratory*

* Statistically significant.

mouse (Snell 1941). The ovarian cycle is polyoestrous, that is, the oestrous cycles recur throughout the breeding season, ovulation occurring spontaneously and not as the result of copulation as in the rabbit. According to Allen (1922, 1923), Parkes (1926*a*), and Merton (1938), the cycles cover a period of 4–6 days, the most usual period being 5 days, as compared with 4–5 days in the rat. In unmated females the corpora lutea of ovulation persist for two, three or four cycles, so that it is impossible to count the number of ova shed during a single oestrous period without staining and cutting sections of the ovary. Copulation takes place during the oestrous period, which usually occurs during the hours of darkness, that is, during the first half of the night, occasionally during the second, and only rarely during the day. A useful indication that mating has taken place is provided by the secretion of the seminal vesicles of the male, which coagulates in the vagina to form the vaginal plug. This remains there for 18–24 hr. and occasionally as long as 48 hr. after mating, before falling out. The ova are fertilized in the upper end of the oviducts, into which they are shed at ovulation. The length of the gestation period in non-suckling mice varies in different strains. Bonhote (Barrett-Hamilton 1910, p. 659) records an exceptionally short period of 13 days, but for non-suckling mice it is described by most workers as being from 19 to 20 days. Some writers give precise data, as follows:

	period of gestation in days						strain
	18	19	20	21	22	mean	
Parkes (1926 <i>a</i> , p. 161)	6	38	3	—	—	18.91	albino
Snell (1941, p. 56)	1	41	51	6	—	19.63	black
	—	10	84	26	3	20.17	dilute brown
	average of means						19.57

For the purposes of this investigation 19.5 days is adopted as the average of gestation. When fertilization takes place during the post-partum oestrus, which occurs about 20 hr. after parturition, the gestation period is prolonged by anything from a day or two to 16 days (Grüneberg 1943), due to delay in implantation. Successful matings at the post-partum oestrus occur regularly in some stocks of mice.

After the ova have been fertilized they pass on into the uterus, where they become implanted at more or less regular intervals throughout its length. Rapid growth now takes place, and it is at this time, 5 days after mating, that the embryos become visible as swellings in the uterus (Snell 1941). It is from the stage of visible pregnancy that the calculations of pregnancy rates and annual productivity have been made, the period of visible pregnancy being counted as 14.5 days. This period is not affected by the lengthening of the gestation period associated with fertilization during the post-partum oestrus, since the prolongation occurs in the pre-implantation stage.

The span of life of laboratory mice has been found by various workers to reach about 3½ years (156–182 weeks), while Metschnikoff and Weismann had an excep-

tional stock where the span of life was as long as 5–6 years (260–312 weeks) (see Backmann 1939). The average length of life is about 90 weeks (Greenwood 1928; Robertson 1926), when, according to Robertson, senility begins to set in. Snell (1941) states that the useful breeding period of most inbred females terminates when they are 10 or 12 months old, after which litters tend to be small and breeding irregular.

It must be borne in mind that all the above-quoted data are derived from laboratory mice, whereas the mice considered in this investigation were living under 'wild' conditions.

2. SOURCES OF SUPPLY AND METHODS OF COLLECTING

The sources of supply of the mice fall under four heads comprising four different environments. These are: (1) cold stores, (2) Ministry of Food buffer depots, (3) corn-ricks, (4) urban—including warehouses, shops, restaurants, and dwelling houses.

In the cold stores, buffer depots and urban environments the most generally effective method of capture was found to be the break-back trap baited with a pinch of rolled oats. The specimens from the cold stores, the Port of London warehouses and most of the buffer depots were obtained by the local rodent officers as the result of their 'trapping out' operations, and were dispatched in tin containers to the Bureau. This method was found to be quite satisfactory except in the hottest months of the year, when the supply of mice fell, as they became unfit for posting. In order to overcome this difficulty the writer found that they could be preserved for at least a week in methylated alcohol or in 25% alcohol without deteriorating, and that when dried in the sun or over the hot pipes for about half an hour, the process made no significant difference to their weight. At higher concentrations it is impossible to distinguish the individual sperms in the cauda epididymidis, so that smears, which are useful criteria of fecundity, became valueless. This, I am informed by Dr J. R. Baker, is caused by the coagulation of the protein in the epididymis into a meshwork with which the spermatozoa become so entangled that they can no longer be distinguished as individual elements.

Most of the urban mice were trapped by the writer in various shops, restaurants and private houses in and around Oxford. The mice from the corn-ricks were also obtained by the writer with the help of Mr H. N. Southern. This necessitates attendance while the ricks are threshed, when the sheaves are lifted up and the mice exposed. In a lightly infested rick the surest and most humane method was thought to be to catch them by hand and kill them by knocking their heads against some hard object; if the infestation was heavy it was found necessary to use sticks or swatters.

Some fairly complete rick populations were obtained. For this purpose it was found necessary to surround the rick with small-mesh ($\frac{3}{8}$ in.) shrimp netting. This was hung over the 30 in. wire-netting fence which is erected round the rick, usually

at a distance of about 6 ft. from the base, in accordance with *The Rats Order* (No. 2) (1940). The shrimp netting was secured to the ground in such a way as to prevent the mice from escaping underneath it. By this means it was possible to kill almost the entire population of a rick. Even under these conditions a certain number of the small mice escaped.

The trapping methods employed in obtaining samples tend to select the heavier weight groups, so that samples of the lower weight groups of trapped mice are not numerically comparable with those from the ricks, where more complete populations were obtained.

3. DESCRIPTION OF ENVIRONMENTS

Cold stores provide environmental conditions which are fairly constant throughout the year. The temperature is permanently at least 17° F below freezing (varying in different stores from -5 to 15° F (-21 to -10° C), according to the purpose for which the store is being used), and the diet is entirely meat. Mice will take beef, mutton or bird, their preference being for the last; in some stores bags of frozen kidneys proved attractive. A crate of feathered pigeons not only affords food, but the feathers provide excellent material for nests, which were seen by the writer when visiting a cold store. They will also make deep holes into the meat, in which they make nests out of the hessian bags with which it is covered. The fur of hares and rabbits is also used as nesting material. The mice are often introduced into the cold stores with the meat. When brought from overseas, the meat is sometimes found to be already infested when removed from the cold stores of the ship. The lighting of cold stores is entirely artificial, and is only on when the goods are being moved, so that for most of the time the mice are living in complete darkness. Mice thrive under these conditions, and exceed in size those from the other environments from which supplies were obtained.

The buffer depots are Ministry of Food storehouses, which may be either perfectly new rat-proof buildings or some large building such as an entertainment hall, school or factory, which has been requisitioned. Practically all the buffer depots from which supplies were obtained fall into the latter category and are scattered all over Britain, even as far north as the Isle of Skye. The buildings are unheated, with wooden floors, brick walls, and rather poor lighting. For the purpose of this investigation only those buffer depots storing sacks of flour and sugar, that is, the vast majority of those from which supplies were received, are included. The flour was white (not 'National') and formed the staple diet of the mice, although sometimes they nibbled at the sugar. The sacks are piled up into large stacks among which they live.

The corn-ricks from which samples were obtained were within about 30 miles of Oxford. The mice live on the grain which they nibble up into characteristically small pieces, thus damaging a lot more than is actually eaten. They are distributed throughout the whole rick from top to bottom and even live and nest in the earth

and decaying matter under the rick. The invasion usually begins at the top, as is so often the case with rat infestations, and then spreads through the whole rick until it is completely riddled by mice. The nests are usually made by eating out a small cavity in the sheaves, about 3 in. in diameter, and lining this with chaff and nibbled ends off bits of straw. Sometimes they are made only of chaff, or they may be in the more compact form of a ball made out of the stems of the corn. As they are loose structures they fall to pieces at the slightest touch, so considerable care was taken to obtain complete litters. Although the mice living inside the rick must be in complete darkness, they come out to the entrance of their tunnels a good deal during the daytime and are often seen darting about on the outside from one hole to another, or sometimes just sitting in the entrance nibbling corn. Mr L. S. V. Venables has provided me with some unpublished observations which he made on internal rick temperatures near Oxford, from 12 February to 2 March 1942. The temperatures inside the rick varied considerably, but did not fall as low or reach such a high level as those outside.

Ricks are usually built during the months of August and September, and for the first couple of months show few signs of mouse infestation, though they may have already been infested by a few which have been living in the fields or neighbouring farm buildings. It also often happens that the mice are carried in the sheaves of corn when they are carted off the field to build into a rick.

The majority of ricks from which samples were obtained were threshed in December and January and so had been standing for 4-5 months. Most ricks are threshed by July, so that there are few records from this time until December when the new ricks are once more noticeably infested. The rick is therefore only a temporary environment.

In all the above environments the mice are actually living in and upon a practically unlimited food supply of a particular kind, which also provides them with cover and a certain amount of protection from predators.

In the *urban habitats* the mice are often obliged to run into the open to obtain their food. This means that in most cases their feeding will be more sporadic than that of mice living in the other environments, and that they are exposed to greater danger from predators. Their food supply tends to be very varied, and from some localities somewhat limited in quantity. The temperature is usually about 'room temperature'.

4. METHODS OF RECORDING AND GROUPING DATA

The details of each mouse were entered on the Copeland-Chatterson 'Paramount' punch cards, size 3 x 5 in. (see figure 1) designed by the Bureau of Animal Population for rat records. This system is most convenient and saves both time and labour.

Each mouse was given a serial number. The localities from which the specimens came were denoted by means of code numbers. The date on which the animal was

caught and the method of killing, whether by break-back trap, hand, poison, or fumigation, was also noted.

All individuals were sexed, and were weighed separately except in the case of complete litters, where the total litter was weighed and divided by the total number of young to give the mean weight of the nestlings. The weighings were read to the nearest hundredth of a gram, and recorded in tenth of a gram units, in such a way that, for example, all measurements from 12.40 to 12.49 g. were grouped as 12.4 g. In the case of pregnant females, the parent and embryos were first weighed as a whole, the uterus and embryos were then removed and weighed to the nearest hundredth of a gram, and the result subtracted from the original weight of the mother.

♂	F	♀	P	Y	AD	UR	1	7	4	2	1	4	2	1	7	4	2	1	7	4	2	1				
							T.	UNITS				TENS				UNITS				T./THOUS.						
							YEARS								WEEKS											
No.																						Testes wt. (mg.)				
Place No.																						Sperms				
Date																						Body length (mm.)				
Sex																						Tail length (mm.)				
Corpora lutea																						Wt. (g.)				
Pregnant																						Wt. embryos (g.)				
No. Embryos																						Skull not kept				
																						Skin not kept				
																						Pelage				
																						Poison				
Pat. Nos. 225069, 297992 c.c., 89336 U.																										
LOCALITY No.																										

FIGURE 1. Punch card and list of data recorded for *Mus musculus*.

The data recorded are as follows:

Serial number	Sex	Condition of pelage
Place number	Weight	Skull, whether kept
Date killed	Body length	Skin, whether kept
Method of killing, poison if any	Tail length	
Female	Male	
Corpora lutea, present or absent	Sperm in cauda epididymidis, present or absent	
Placental sites and spots in uterus if present	Weight of testis	
Whether pregnant		
Number of embryos		
Weight of embryos		

Top holes: ♂, fecund; ♀, pregnant; young, adult; urban, rural; coding for year ('1' onwards) and week (fixed arbitrarily in 52 units for a basic year); coding for locality number; spare numbers 1-32, for method of capture or death, weight groups, etc.

In tabulating the samples, the mice were allocated to weight groups of 2.5 g., the lowest weight group being from 0.5 to 2.4 g., as naked nestlings always weighed over 0.5 g. For some purposes, e.g. when considering the percentage number of adult females pregnant throughout the year, larger weight groups of 5.0 g. were used, which is much more satisfactory when some of the samples are rather small.

The condition of the reproductive organs of the females was determined by making a median incision on the ventral surface of the abdomen and examining the ovaries and uterus *in situ*. The presence of spots or placental sites in the wall of the uterus was noted, and if the female was pregnant the number of embryos in the left and right horns recorded.

The condition of the reproductive organs of the males was determined by opening the left scrotal sac and removing the testis, which was weighed to the nearest milligram, without any investing tissue or epididymis. The cauda epididymidis was teased out on a slide and examined microscopically for sperm. Usually these occurred in large numbers or not at all; but there were some cases in which a few spermatozoa only were visible, this condition being usually associated with a testis weight of between 20 and 30 mg. It is known that it is necessary for a large number of sperms to be present round the egg if successful fertilization is to take place. Therefore, although it is difficult to assess whether some of the intermediate forms were fecund or not, it is more probable that they were not, and they were recorded as non-fecund. It was found that a testis weight of 30 mg. or over was almost invariably associated with fecundity, that is, with a large number of sperms in the cauda epididymidis. Below a weight of 20 mg. the cauda was nearly always without sperms, that is to say, the males were non-fecund. Occasionally, however, testis weights between 20 and 30 mg. were associated with large numbers of sperms and the animals therefore fecund. After the examination of over a thousand individuals, however, the writer found that if the tubules in the cauda were visible to the naked eye they invariably contained quantities of sperms and that therefore the individual would be fecund. This greatly simplified the routine work, as the cauda could be examined *in situ* with the naked eye. Sperm smears were made in any doubtful cases and examined microscopically. Moreover, it followed that further weighing of the testis was quite unnecessary. It was also found that below a body weight of 9 g. the males were invariably non-fecund, and so could be recorded as such without further examination.

Any additional points of interest, such as unusual pelage colour, or the presence of some disease, were also recorded on the punch card.

In subsequent grouping of the data the year was divided into thirteen 4-weekly periods, commencing from 8 January 1942, the date upon which the first specimens were killed, and the weeks were numbered serially 1-52, the first week being 8-14 January. This method obviously provides a much better basis for comparison than the variable calendar months.

5. SIZE OF LITTER

The size of litter depends upon two main factors, the number of eggs fertilized and the amount of foetal elimination. Brambell (1928) records for a large number of laboratory-bred albino mice an average number of maturing follicles of 9.3, associated with an average size of litter of 6.34, i.e. a foetal elimination of approximately one-third.

Data regarding the size of litter have been given by various workers. Robertson (1916) found an average of 5.15 associated with the high variability of 42.8%; Parkes (1926*b*) found the average litter size of 1872 albino mice to be 6.18; Merton (1938) found an average size of 7.0 out of 504 litters of albino mice; and Gates (1925) an average of 7.4 with a range of 2-12 for 106 litters of a random-bred strain of laboratory mice.

TABLE 1. NUMBER OF YOUNG PER COMPLETE LITTER, AND THE SEASONAL FREQUENCY DISTRIBUTION OF THE LATTER

number of young	8 Jan.- 4 Mar. 1943	5 Mar.- 29 Apr. 1943	30 Apr.- 24 June 1942	25 June- 19 Aug. 1942	20 Aug.- 14 Oct. 1942	15 Oct. 1942- 8 Jan. 1943	total
2	—	1	—	—	—	—	1
3	—	1	—	—	—	3	4
4	4	2	—	1	3	6	16
5	2	1	—	1	1	4	9
6	2	—	—	2	2	7	13
7	3	—	—	2	—	1	6
8	—	2	—	—	—	—	2
9	1	2	—	—	—	—	3
10	—	—	—	—	—	—	—
11	2	—	—	—	—	1	3
12	—	—	—	—	—	1	1
13	—	—	—	—	—	1	1
total	14	9	—	6	6	24	59
average	6.43	5.78	—	5.83	4.83	5.75	5.83 ± 0.31

In the present investigation only a few nests were taken from environments other than ricks. From the latter there were obtained 59 complete litters containing 344 mice. These were taken between April 1942 and April 1943. The average number per litter is 5.83 ± 0.31 , which is about half a mouse below the average of the data for the laboratory mice given above; there is a wide range in the size of litter, from 2 to 13, which is in agreement with the records of Robinson and of Gates, assuming that communal nests were not included. Nests containing a large number of young are often communal. A number of these were found in the ricks, the young belonging to two or three litters. The particulars are given in table 1.

Most ricks are threshed before June, and the new ones have not been erected long enough to become thoroughly infested by October, so that only a few complete litters were obtained during the summer months. Any significance which might be thought to be attached to the absence of larger litters during this period is discounted by the small number of litters taken.

6. SEX RATIO

The proportion of males and females in the total of 5468 wild mice (*M. musculus*) examined by the writer during the 52 weeks from 8 January 1942 to 7 January 1943, from all four environments taken together, is 2627 males and 2841 females. The ratio is 92.5 males to 100 females, a male percentage of 48.04 (tables 2-4).

(a) Sex ratio and environment

The distribution of the total number among the several environments is given in table 2. The figures as a whole show a preponderance of females, but that this is entirely dependent upon the numbers for the ricks. The χ^2 test (Fisher 1936) does not show any statistically significant difference in the distribution of the sexes as between urban and buffer depots, urban and cold stores, or buffer depots and cold stores; and in all these three environments the percentages may be looked upon as approximations to 50 %. When the numbers for the three groups are added together the approximation becomes exceedingly close (table 2). In the case of

TABLE 2. TOTALS AND PERCENTAGES OF THE SEXES
FOR THE DIFFERENT ENVIRONMENTS

	male	female	total	male %	female %
urban	553	514	1067	51.83	48.17
buffer depots	580	570	1150	50.43	49.57
cold stores	587	631	1218	48.19	51.81
total	1720	1715	3435	50.07	49.93*
ricks	907	1126	2033	44.61	55.39
total	2627	2841	5468	48.04	51.96

* Calculated from totals.

the ricks on the other hand the ratio is clearly different; and the χ^2 test applied to 2×2 contingency tables, using Yates's correction for continuity, indicates significant statistical differences in sex distribution as between ricks and domestic ($\chi^2 = 14.33$ with one degree of freedom, giving $P = 0.01$) and between ricks and buffer depots ($\chi^2 = 9.76$ with one degree of freedom, giving $P = 0.01$), and a lesser difference between ricks and cold stores, which is on the border between significance and non-significance ($\chi^2 = 3.79$ with one degree of freedom, giving P of 0.05).

TABLE 3. SEASONAL DISTRIBUTION OF SEX RATIOS FOR THREE DIFFERENT ENVIRONMENTS

	urban		buffer depots		cold stores		χ^2	n	P
	female	male %	female	male %	female	male %			
8 Jan.-4 Feb.	10	3	37	45	98	85	4.97	2	0.10-0.05
5 Feb.-4 Mar.	11	20	98	103	35	26	3.98	2	0.20-0.10
5 Mar.-1 Apr.	45	40	21	29	76	81	1.51	2	0.50-0.30
2 Apr.-29 Apr.	15	13	99	93	87	80	0.04	2	0.98
30 Apr.-27 May	55	61	92	116	44	28	6.14	2	nearly 0.05
28 May-24 June	31	32	15	16	29	20	1.36	2	0.50 approx.
25 June-22 July	50	47	5	4	15	14	0.01	1	0.90
23 July-19 Aug.	14	26	3	5	22	25	1.27	1	0.30-0.20
20 Aug.-16 Sept.	29	43	35	28	54	55	3.25	2	0.20
17 Sept.-14 Oct.	21	28	116	84	22	21	3.87	2	0.20-0.10
15 Oct.-11 Nov.	65	60	33	39	36	44	1.21	2	0.70-0.50
12 Nov.-9 Dec.	106	108	16	18	19	14	0.89	2	0.70-0.50
10 Dec.-7 Jan.	62	72	—	—	94	94	0.30	1	0.70-0.50
totals	514	553	570	580	631	587	28.80	23	0.20 approx. not sig.
χ^2 as evidence re	14.12		11.67		8.15				
seasonal homogeneity	11		10		12				
n	0.20 approx.		0.30 approx.		0.80-0.70				
P	not signif.		not signif.		not signif.				

(b) Sex ratio and season.

The ratio between the sexes does not undergo seasonal change in any of the three environments—urban, buffer depots, cold stores—the χ^2 test indicating that the differences in sex ratio throughout the season are, for each of these environments taken separately, not greater than may reasonably be referred to chance (table 3). Similarly, there is no difference of statistical significance between the samples from the three environments named, for any one and the same 4-weekly period; in one case only (30 April–27 May) does the probability that the differences recorded are due to chance fall as low as 5%.

The rick samples (table 4) are not so complete, but they support the theory of seasonal consistency, while confirming generally the evidence of the totals as to numerical preponderance of females in the ricks, in contrast to the numerical equality between the sexes found elsewhere. Parkes (1924*a*), however, found that the sex ratio varied with the breeding season in laboratory-bred mice; the proportion of males was lowest in the height of the breeding season, i.e. May–June, and highest at the extreme end, i.e. October–December. A similar phenomenon has been observed among dogs (Heape 1907) and among albino rats (King & Stotsenburg 1915).

TABLE 4. SEASONAL DISTRIBUTION OF SEX RATIOS FOR RICK MICE

	females	males	male %
8 Jan.–4 Feb.	—	—	—
5 Feb.–4 Mar.	—	—	—
5 Mar.–1 Apr.	40	24	37.50
2 Apr.–29 Apr.	48	45	49.52
30 Apr.–27 May	5	7	43.91
28 May–24 June	64	42	39.62
25 June–22 July	103	97	48.50
23 July–19 Aug.	—	—	—
20 Aug.–16 Sept.	—	—	—
17 Sept.–14 Oct.	125	98	43.91
15 Oct.–11 Nov.	4	3	43.91
12 Nov.–9 Dec.	—	—	—
	389	316	44.82
10 Dec.–7 Jan.	737	591	44.50
	1126	907	44.61

χ^2 as evidence re seasonal homogeneity = 4.68

n

= 5

P

nearly 0.50, i.e. not significant

The question arises as to whether the ratio found in the rick samples represents a biological phenomenon or whether it is due to the method of sampling, a larger number of males escaping than of females. Against the latter suggestion is the fact that the numbers for 10 December–7 January are made up of several rick populations, which, though not quite complete, are fairly so, and the same ratio

obtains here as is the case on the average for the smaller samples. In this connexion it is of interest that the results of observations by Naumov (1936) among voles, *Microtus arvalis*, living in natural conditions, agreed with those of Rörig and Krampe who had previously found that during the summer and autumn, i.e. the breeding season, there were more females than males.

(c) *Sex ratio and weight*

It was found by members of the Bureau of Animal Population that among samples of rats obtained from corn-ricks, there was a slight preponderance of males among nestlings and animals of lighter body weight, whereas in the heavier weight classes females outnumbered males. In order to find out whether a similar switch over occurs among mice the data have been grouped as in table 5.

TABLE 5. SEX RATIO IN RELATION TO WEIGHT

classes	wt. group g.	urban			buffer depots		
		male	female	male %	male	female	male %
(a) nestlings	below 5	19	21	47.50	8	10	44.44
(b) non-fecund (predominantly)	5.0-9.9*	96	78	55.17	92	80	53.49
(c) lighter fecund (predominantly)	10.0-17.4*	310	253	55.06	252	211	54.43
(d) heavier fecund	17.5*	128	162	44.33	228	269	45.88
totals		553	514	51.83	580	570	50.43
(c) and (d) combined		438	415	51.41	480	480	50.00
		cold stores			ricks		
		male	female	male %	male	female	male %
(a) nestlings	below 5	31	37	45.59	169	207	44.95
(b) non-fecund (predominantly)	5.0-9.9*	101	92	52.33	211	240	46.78
(c) lighter fecund (predominantly)	10.0-17.4*	229	183	55.58	336	416	44.68
(d) heavier fecund	17.5*	226	319	41.41	151	189	44.41
totals		587	631	48.19	867	1052	45.18
(c) and (d) combined		455	502	47.54	487	605	44.60

The smaller numbers given for the rick mice in this table as compared with the numbers in tables 2 and 3 are due to the omission of certain mice which were examined alive.

* For each class the cold store mice are approximately 2.5 g. heavier.

Among the nestlings of all environments there is a preponderance of females, though except for the ricks the numbers are not large. In the non-fecund active mice the preponderance remains with the females in the rick samples but passes to the males for the other environments. In the lighter fecund class the same sex is favoured for each environment as in the non-fecund class. In the heavier fecund class a switch-back in favour of the females which is shown by the χ^2 test to be of

statistically significant proportions, occurs in urban, buffer depot and cold-store samples, so that in all environments the females now once more predominate. Cole & Hart (1938) state that pregnancy stimulates skeletal and tissue growth in the rat, so that a similar suggestion may be made for the mouse, to account for the switch-back referred to. Weight is a rough criterion of age, but were it possible to group the animals accurately according to age some of the females in the fourth class would on this view have to be put back into the third. It will be noted that for the combined fecund class numbers the sexes approximate to numerical equality in the urban, buffer depot and cold-store mice, particularly the two former. The persistent numerical predominance of the females at all weights in the rick mice is noteworthy.

The sex ratio of laboratory-bred albino mice was found by MacDowell & Lord (1925) to be equality; they obtained 261 males and 262 females from 106 litters. Equality was also found by Gates (1925) for 700 laboratory-bred young from 106 litters. Parkes (1924*a*), dealing with over 1000 albino mice from 157 litters, records a ratio of 118 males to 100 females, that is to say 54.13% males, and refers to Darling as finding a rate of 133.8 males to 100 females for 255 white mice, a male percentage of 57.23. Chappellier (1937) records for 207 post-nestling 'common mice' living under natural conditions a proportion of 110 males and 96 females, a male percentage of 53.30.

7. WEIGHT DISTRIBUTION

Tables 6-8 show the weight distribution of mice obtained from all environments between 8 January 1942 and 7 January 1943.

On reviewing the weight distribution of active mice (table 6) it becomes clear that the trapping methods employed in the cold store, buffer depot, and urban environments are selective, a large number of the small mice, those in the lighter weight groups, failing to be caught. The weight distribution in the rick samples is much closer to what might be expected in a normal population and also from the pregnancy rates, though even here it appears that a certain percentage of the younger members of the population may have escaped; one would have expected higher figures in the lower weight groups. The weight distribution of active mice from three fairly complete rick populations is given in table 8, and shows such a tendency.

As the samples from cold stores, buffer depots, and urban habitats were all obtained by trapping, their weight distributions are comparable with each other, but not with those from the ricks. Their mean weights are the means of the trapped members of the population and are therefore biased in favour of the heavier weight groups.

The mean weights of the cold-store mice are markedly heavier than the means for the other environments. Their greater weight is due to a general increase in size, not to excess fat. The χ^2 test between the distributions of the weights of fecund

TABLE 6. WEIGHT DISTRIBUTION OF ACTIVE MICE AND FEMALE PERCENTAGES IN THE VARIOUS WEIGHT GROUPS,
FROM 8 JANUARY 1942 TO 7 JANUARY 1943

weight group g.	urban			buffer depots			cold stores			ricks		
	number males	number females	% females	number males	number females	% females	number males	number females	% females	number males	number females	% females
5.0-}	38	29	44.83	31	32	46.78	28	26	46.09	107	148	53.22
7.5-}	58	49		60	48		34	27		104	92	
10.0-}	68	74	47.01	76	69	47.50	39	39	52.20	123	134	54.46
12.5-}	109	83		71	64		48	56		112	147	
15.0-}	133	96	43.49	105	78	44.50	75	55	44.65	101	135	58.68
17.5-}	84	71		127	108		106	72		87	132	
20.0-}	32	42	63.56	71	91	58.82	104	74	47.53	43	46	46.61
22.5-}	11	33		27	49		66	80		20	9	
25.0-}	1	15	93.75	2	16	87.50	39	82	75.15	1	2	50.00
27.5-}	—	0		1	5		13	45		—	—	
30.0-}	—	1	100.00	1	—		4	25	90.00	—	—	
32.5-}	—	—		—	—		—	11		—	—	
35.0-}	—	—		—	—		—	1		—	—	
37.5-}	—	—		—	—		—	0	100.00	—	—	
40.0-}	—	—		—	—		—	1		—	—	
mean	534	493		572	560		556	594		698	845	
standard error of mean	13.19	14.12		14.40	15.23		16.88	18.95		11.92	11.97	
coefficient of variation	± 0.18	± 0.23		± 0.20	± 0.23		± 0.24	± 0.28		± 0.18	± 0.16	
	32.39	35.97		33.93	35.19		33.56	36.37		40.83	39.79	

TABLE 7. WEIGHT DISTRIBUTION OF FECUND MICE AND FEMALE PERCENTAGES IN THE VARIOUS WEIGHT GROUPS, FROM 8 JANUARY 1942 TO 7 JANUARY 1943

weight group g.	urban		buffer depota		cold stores		ricks	
	number males	number females	number males	number females	number males	number females	number males	number females
		% females		% females		% females		% females
7.5-	8	20	0	25	1	4	0	14
10.0-	42	72	23	66	5	23	45	113
12.5-	97	83	48	61	26	53	84	145
15.0-	127	96	92	78	58	53	98	135
17.5-	81	71	112	108	98	72	87	132
20.0-	31	42	63	91	100	74	43	46
22.5-	10	33	24	49	66	80	20	9
25.0-	1	15	2	16	39	82	1	2
27.5-	—	—	1	5	11	45	—	—
30.0-	—	—	1	—	4	25	—	—
32.5-	—	—	—	—	—	11	—	—
35.0-	—	—	—	—	—	1	—	—
37.5-	—	—	—	—	—	0	—	—
40.0-	—	—	—	—	—	1	—	—
mean	397	433	366	499	407	524	378	596
standard error of mean	14.82	15.19	16.61	16.09	19.36	20.47	15.42	14.35
coefficient of variation	± 0.16	± 0.21	± 0.18	± 0.20	± 0.20	± 0.25	± 0.17	± 0.14
	21.52	29.16	20.19	27.72	20.33	28.28	22.04	23.81

TABLE 8. 'COMPLETE' ACTIVE POPULATIONS FROM THREE RICKS, (a), (b) AND (c); AND FEMALE PERCENTAGES IN THE VARIOUS WEIGHT GROUPS, CALCULATED FROM THE TOTAL

weight group	(a)		(b)		(c)		total		%
g.	males	females	males	females	males	females	males	females	females
5.0-}	12	23	15	16	7	8	34	47	52.38
7.5-}	13	10	6	6	7	3	26	19	
10.0-}	12	11	2	2	9	6	23	19	47.67
12.5-}	7	10	7	5	8	7	22	22	
15.0-}	18	10	7	6	12	12	37	28	57.76
17.5-}	8	11	1	8	3	8	12	27	
20.0-}	—	4	1	8	—	1	1	13	93.75
22.5-}	—	1	—	1	—	—	—	2	
	70	80	39	52	46	45	155	177	
mean	11.07	10.87	9.49	11.92	11.09	12.50	10.68	11.53	
standard error of mean	± 0.50	± 0.57	± 0.72	± 0.82	± 0.57	± 0.65	± 0.34	± 0.39	
coefficient of variation	38.09	46.73	47.04	49.83	34.75	35.02	39.65	45.40	

females from the cold stores and buffer depots gives $\chi^2 = 159.434$, which, with $n = 10$, gives a value of P much less than 0.01, indicating significant difference. The greater weight probably reflects the effect of conditions of life in cold stores.

The difference between the distributions of weights of buffer depot and urban samples is also of statistical significance; again comparing the fecund females $\chi^2 = 30.922$, which, for 7 degrees of freedom, gives P less than 0.01. However, it seems doubtful whether this significance is biological; it may merely be the result of differences in sampling.

The heaviest female recorded weighed 40.40 g. and is from a cold store. This is 10 g. heavier than the heaviest female from any other habitat, the next heaviest being one from an urban habitat and weighing 30.40 g. It appears also to be the heaviest wild specimen of *Mus musculus* so far recorded. The heaviest male is also from a cold store; it weighed 31.0 g., only a little heavier than the next heaviest, from a buffer depot, which was 30.3 g.

Tables 6-8 also give the coefficients of variation. It will be seen that the coefficients of variation for females from all habitats excluding ricks are very similar. The same is true of the males. On the other hand, the variability is greater in the females than in the males. In the ricks, by way of contrast, there is no substantial difference in variability between male and female. The query arises as to whether the difference between male and female variability in the other habitats reflects an occurrence in nature or is merely an indication of selective trapping.

The percentage of females for each weight group has been included in the tables. The trend of these percentages in relation to increasing weight, as seen in table 6, is in line with the discussion in the section on sex ratio and weight above. Table 7

shows that among fecund mice, females predominate at both the lighter and heavier ends of the scale, whereas in certain intermediate weight groups (15–17.5 g.) males are more numerous than females. The predominance of lighter females in the lower weight groups indicates that fecundity is reached by the females at a lighter weight than is the case in the males.

8. FECUNDITY

(a) *Criteria of fecundity*

The index of fecundity in the female which is adopted in the present investigation as being in the view of the writer the most satisfactory, is the presence of corpora lutea in the ovary. The condition of the vaginal orifice alone, whether perforate or imperforate, is not a reliable criterion as to whether the individual is capable of becoming pregnant. Parkes (1925) found an interval of a week between the establishment of the vaginal orifice and the first ovulation, although Engle & Rosasco (1927) found, as the result of the examination of 100 female albino mice, that ovulation usually occurs within 24 hr. of its opening. There is thus a margin of uncertainty in addition to which the vagina may be forced open accidentally during examination. The condition of the uterus is a useful indication of ovulation, as after the first oestrus it becomes enlarged.

The criterion of fecundity applied to the males is the presence of a fairly large number of sperm in the cauda epididymidis, which, as has already been stated, can be determined with the naked eye.

(b) *Weight and fecundity*

The attainment of fecundity in relation to weight is indicated in tables 9 and 10.

Supplementary to the tables the weights of the lightest fecund individuals for each environment may be recorded, as follows: cold stores, female 8.0, male 9.0 g.; buffer depots, female 7.9, male 10.3 g.; urban, female 8.2, male 9.0 g.; ricks, female 8.6, male 10.4 g.

Summarizing these particulars of fecundity the following points will be noted. No females were found to be mature below the 7.5 g. weight group, and very few males below the 10.0 g. group; the lightest fecund male recorded for each environment is heavier than the lightest fecund female; and the weight at which fecundity becomes general is for each environment a heavier one for the males than for the females. As compared with the mice from the three environments, those from the cold stores exhibit a retardation of fecundity in relation to weight. For the three environments, urban, buffer depots, and ricks, females weighing 15 g. or over were always found to be fecund; for cold-store mice the same was true for mice of 17.5 g. or over.

Data for the relation of the attainment of fecundity to weight are scarce. Engle & Rosasco (1927) found that among 100 female albino mice the weight at which the vagina opened ranged from 10 to 16 g., with a median of 13 g. This is generally

TABLE 9. NUMBER OF FECUND FEMALES IN RELATION TO WEIGHT

weight group g.	cold stores			buffer depots			urban			ricks		
	non-		% fecund	non-		% fecund	non-		% fecund	non-		% fecund
	fecund	fecund		fecund	fecund		fecund	fecund		fecund	fecund	
7.5-	4	23	14.81	25	23	52.08	20	29	40.82	14	78	15.22
10.0-	23	16	58.97	66	3	95.65	72	2	97.30	113	21	84.33
12.5-	53	3	94.64	61	3	95.31	83	0	100.00	145	2	98.64
15.0-	53	2	96.36	78	0	100.00	96	0	100.00	135	0	100.00
17.5-	72	0	100.00	108	0	100.00	71	0	100.00	132	0	100.00
20.0-	74	0	100.00	91	0	100.00	42	0	100.00	46	0	100.00
22.5-	80	0	100.00	49	0	100.00	33	0	100.00	9	0	100.00
25.0-	82	0	100.00	16	0	100.00	15	0	100.00	2	0	100.00
27.5-	45	0	100.00	5	0	100.00	—	—	—	—	—	—
30.0-	25	0	100.00	—	—	—	1	0	100.00	—	—	—
32.5-	11	0	100.00	—	—	—	—	—	—	—	—	—
35.0-	1	0	100.00	—	—	—	—	—	—	—	—	—
37.5-	—	—	—	—	—	—	—	—	—	—	—	—
40.0-	1	0	100.00	—	—	—	—	—	—	—	—	—
9.0-9.9	2	8	20.00	15	5	75.00	15	8	65.22	12	34	26.09

TABLE 10. NUMBER OF FECUND MALES IN RELATION TO WEIGHT

weight group g.	cold stores			buffer depots			urban			ricks		
	non-		% fecund	non-		% fecund	non-		% fecund	non-		% fecund
	fecund	fecund		fecund	fecund		fecund	fecund		fecund	fecund	
7.5-	1	33	2.94	0	60	0.00	8	50	13.79	0	104	0.00
10.0-	5	34	12.82	23	53	30.26	42	26	61.76	45	78	36.59
12.5-	25	23	52.08	48	23	67.61	97	12	88.99	84	28	75.00
15.0-	58	17	77.33	92	13	87.62	127	6	95.49	98	3	97.03
17.5-	98	8	92.45	112	15	88.19	81	3	96.43	87	0	100.00
20.0-	100	4	96.15	63	8	88.73	31	1	96.88	43	0	100.00
22.5-	66	0	100.00	24	3	88.89	10	1	90.91	20	0	100.00
25.0-	39	0	100.00	2	0	100.00	1	0	100.00	1	0	100.00
27.5-	11	2	84.61	1	0	100.00	—	—	—	—	—	—
30.0-	4	0	100.00	1	0	100.00	—	—	—	—	—	—
11.5-12.4	4	6	40.00	13	21	38.24	18	10	64.29	26	27	49.06

similar to the findings in the present investigation. For environments other than cold stores a few mature females were found which weighed about 8 g. (the lightest weighing 7.9 g.), and at 15 g. all were mature, maturity being thus reached at rather a lower weight than in the albino mice of Engle & Rosasco. It would appear that a closer approximation to their figures is given by the mice from the cold stores. They remark that in the laboratory mice with which they worked, a range of weight from 10 to 16 g. corresponded to an age from 28 to 49 days.

9. FERTILITY

The degree of fertility among the females may be measured either by the number of adults pregnant or by the number of embryos per adult female. Advantages and limitations apply to both methods, so that both have been used in this work.

A. *Pregnancy rates*

Pregnancy rates correspond pretty accurately with litter rates. It does not appear likely that all embryos are often resorbed. In 377 pregnancies only one case of this was found (see p. 277). J. S. Perry (1945) only found three cases of the total absorption of all embryos out of 131 pregnancies in wild rats.

(a) *Pregnancy rates and weight*

In absence of knowledge of the age of the mice, the pregnancy data are grouped in relation to weight (table 11A). Besides illustrating the increase in percentage pregnancy associated with increase in weight, table 11 also shows that pregnancy percentages in relation to weight differ in different environments. The buffer depot mice occupy an intermediate position between those from cold stores and those from ricks, a higher proportion of the rick mice becoming pregnant at a somewhat lighter weight, and of cold store mice at a considerably heavier weight. The scale adopted in the table makes it appear that the difference in weight between cold-store mice and those from the buffer depots in relation to a similar frequency of pregnancy is some 5 g., and analysis by means of a scale of smaller units does not materially affect this conclusion (see table 11B). There may be an association between these differences and temperature. The ricks afford the warmest environment, while the cold stores are uniformly the coldest. Another factor to be borne in mind is the lesser liability to disturbance of the mice in the ricks as compared with those in the other environments, particularly the urban.

The lighter members of the urban samples show very similar pregnancy rates to those for mice of similar weights from the buffer depots, but the pregnancy rate does not rise with weight in the appreciable way in which it does among mice of the other environments.

(b) *Seasonal pregnancy; and correction for weight*

In studying the seasonal incidence of pregnancy by crude percentages an error might be introduced by differences in the weight composition of the samples. In

TABLE 11A. PREGNANCY PERCENTAGES OF FECUND FEMALES IN RELATION TO WEIGHT FOR MICE FROM DIFFERENT ENVIRONMENTS FROM 8 JANUARY 1942, TO 7 JANUARY 1943

weight group g.	buffer depots		urban		ricks		cold stores	
	number	% pregnant	number	% pregnant	number	% pregnant	number	% pregnant
7.5-	91	2-20	92	6-52	81	8-64	27	0-00
12.5-	139	41	179	25-50	196	42-35	106	9-43
17.5-	199	79	114	39-70	170	50-59	146	23-29
22.5-	65	32	47	49-23	21	66-67	162	32-10
27.5-	5	4	1	80-00	—	—	70	45-71
32.5-	—	—	—	—	—	—	12	83-33
37.5-	—	—	—	—	—	—	1	100-00
total	499	158	433	95	468	190	524	139

TABLE 11B. PREGNANCY PERCENTAGES OF FECUND FEMALES IN RELATION TO WEIGHT FOR MICE FROM DIFFERENT ENVIRONMENTS FROM 8 JANUARY 1942, TO 7 JANUARY 1943

weight group g.	buffer depots		urban		ricks		cold stores	
	number	% pregnant	number	% pregnant	number	% pregnant	number	% pregnant
7.5-	25	0	20	1	8	0-00	4	0-00
10.0-	66	2	72	5	73	9-66	23	0
12.5-	61	16	83	14	89	32-58	53	4
15.0-17.5	78	25	96	31	107	50-47	53	6
totals	230	43	271	51	277	90	133	10

order to overcome this difficulty the standardized percentages were calculated for each 4-weekly period by the 'indirect' method. The 'observed' number pregnant in each 4-weekly period was divided by the number 'expected' to be pregnant. This 'expected' number is calculated on the assumption that the average weight-specific pregnancy rates observed over the whole period were actually in operation during each 4-week period. The ratio so obtained is multiplied by the percentage over the whole period of fecund mice of all weights which were pregnant.

$$\frac{\text{observed no. of pregnant for given 4-week period}}{\text{expected no. of pregnant for same 4-week period}} \times \frac{\text{average \% pregnant for whole period (one year), as derived from the totals.}}{1} = \text{standardized \% P.}$$

Thus (table 12) the crude percentage pregnancy for buffer depot mice for the 4-week period 8 January to 4 February, is 25.71, and the standardized percentage (25.64) is obtained as follows:

WEIGHT DISTRIBUTION OF FECUND FEMALES FROM BUFFER DEPOTS
8 JANUARY 1942 TO 7 JANUARY 1943

weight group g.	N.	P.	ratio P./N.		4-week period 8 Jan.-4 Feb.		
					N.	'expected' P.	observed P.
7.5-	91	2	0.0220	x	6	0.1320	0
12.5-	139	41	0.2950	x	8	2.3600	1
17.5-	199	79	0.3970	x	18	7.1460	0
22.5-	65	32	0.4923	x	3	1.4769	2
27.5-	5	4	0.8000	x	0	—	0
	499	158				11.1149	9

$$\frac{\text{total P}}{\text{total N}} = 0.3166 \quad \cdot \quad \frac{9}{11.1149} \times 0.3166 \times 100 = 25.64$$

= standardized % P.

Table 12, constructed in this way, gives the standardized pregnancy percentages for each 4-week period for fecund mice for each of the four environments. The material is set out in graphic form in figure 2.

Table 12 shows that the standardized percentages do not differ materially from the crude percentages, which indicates a general similarity in weight distribution in each 4-week period. It will also be noted that breeding takes place freely in all environments through the year (see figure 2). As the weight distribution of the cold-store mice is so different from the others they must be considered separately, and will be discussed later.

Although it appears from the graph (figure 2) that there might be some seasonal differences in the pregnancy rates of the buffer depot and rick mice, the χ^2 test for each environment considered separately throughout the year gives a value of P which is non-significant. The only indication, and that not a very definite one, of

TABLE 12. SEASONAL DISTRIBUTION OF PREGNANCY IN MICE FROM FOUR DIFFERENT ENVIRONMENTS

N. = number of fecund females. P. = number pregnant. Stand. = standardized.

4-week period	urban			buffer depots			ricks			cold stores		
	N.			N.			N.			N.		
	% P.	stand. % P.	crude % P.	% P.	stand. % P.	crude % P.	% P.	stand. % P.	crude % P.	% P.	stand. % P.	crude % P.
8 Jan.-4 Feb.	8	2	21.05	35	9	25.71	84	31	37.35	61	14	22.95
5 Feb.-4 Mar.	11	2	22.43	72	17	23.61	58	25	43.10	34	5	14.71
5 Mar.-1 Apr.	36	10	26.00	17	4	23.53	38	16	44.44	64	16	25.00
2 Apr.-29 Apr.	14	3	23.56	88	39	44.32	24	9	37.50	72	18	25.00
30 Apr.-27 May	48	12	25.00	87	28	32.18	5	2	27.78	41	8	19.51
28 May-24 June	25	6	24.00	16	5	36.36	13	3	45.61	23	4	17.39
25 June-22 July	31	10	32.26	5	2	32.64	57	26	43.67	9	3	25.93
23 July-19 Aug.	10	2	19.45	32	15	46.88	—	—	—	27	7	21.47
20 Aug.-16 Sept.	26	5	26.32	105	25	23.81	34	19	55.88	45	18	31.75
17 Sept.-14 Oct.	19	5	18.97	27	8	29.63	—	—	—	63	20	27.93
15 Oct.-11 Nov.	58	11	15.10	14	5	35.71	—	—	—	34	10	29.79
12 Nov.-9 Dec.	93	14	24.07	—	—	—	157	59	37.58	47	14	35.37
10 Dec.-7 Jan.	54	13	25.09	—	—	—	—	—	—	13	4	35.87
	433	95		499	158		468	190		524	139	

$\chi^2 = 4.5327$
 $n = g$
 $P = 0.90 - 0.80$ (non-sig.)

$\chi^2 = 13.6396$
 $n = 9$
 $P = 0.20 - 0.10$ (non-sig.)

$\chi^2 = 7.0197$
 $n = 7$
 $P = 0.50 - 0.30$ (non-sig.)

$\chi^2 = 16.8071$
 $n = 9$
 $P = 0.10 - 0.05$ (non-sig.)

an intensified breeding period, is for the mice from the buffer depots during the summer months. The continuity of breeding throughout the year is in agreement with the statement of Sviridenko (1934) that the house-mouse in field conditions in Russia is 'capable of reproduction among natural surroundings the whole year round' and the statement in Barrett-Hamilton (1910, p. 659), that 'in comfortable surroundings in presence of food young are born in every month of the year'. It is, however, in contrast with the observations of Parkes (1924*b*) on laboratory-bred albino mice. He found that there was a breeding season, slightly in favour of the summer months, which began in March, reaching its highest point in May or June.

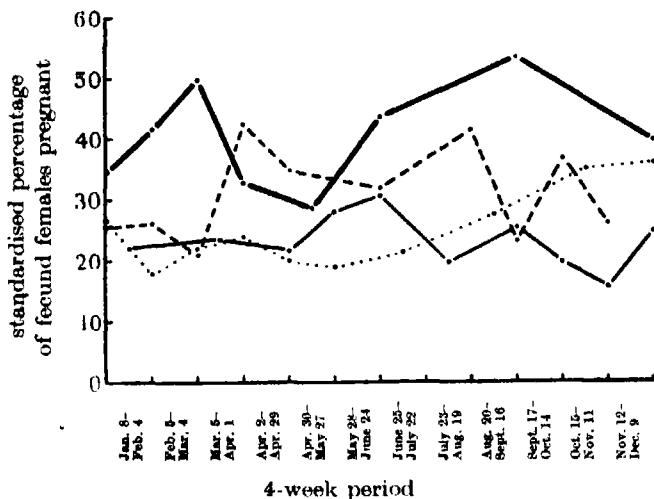


FIGURE 2. Seasonal distribution of pregnancy in fecund mice from four different environments. —•— cornricks, --- flour depots, — — — urban, cold stores.

(c) *Comparison between the samples from different environments*

The question arises as to whether the differences in pregnancy rate which occur between the mice from different environments are of sufficient significance to indicate different rates of breeding attributable to different environmental conditions. To test this the data from the buffer depot, urban and rick environments are compared for each 4-week period. The buffer depot and urban mice show no significant differences ($\chi^2 = 11.579$, $n = 11$, $P = 0.50-0.30$) and may therefore be grouped as 'non-rick' mice with which the rick mice are compared. On three occasions the rate for the rick mice is significantly higher than that for the 'non-rick' (5 Feb.-4 Mar., $\chi^2 = 4.928$, $n = 1$, $P = 0.05-0.02$; 5 Mar.-1 Apr., $\chi^2 = 4.821$, $n = 1$, $P = 0.05-0.02$; 17 Sept.-14 Oct., $\chi^2 = 13.935$, $n = 1$, $P = 0.01$), and as can be seen from figure 2 the standardized percentage of fecund females pregnant is nearly always at a higher figure than that for the other environments.

It has already been shown (tables 6 and 11 A, B) that cold-store mice stand well apart from those from other habitats in being heavier, but putting aside the resulting

difference in incidence of pregnancy at similar weights, there is no marked dissimilarity between the pregnancy rates of cold-store and other mice. There is the same tendency to the association of increased pregnancy percentage with increase in weight, breeding continues throughout the year, and (table 12) fluctuations of statistical significance in the intensity of breeding from season to season are not revealed by the χ^2 test. It will be seen indeed by reference to table 12 and figure 2 that the standardized percentage pregnancy curve for cold-store mice runs remarkably closely with that for domestic samples. The behaviour of the cold-store mice is of interest as it might have been expected that the pregnancy rate would have been markedly affected by the continuous low temperature (below freezing) and the meat diet.

(d) *Annual productivity*

The average proportion of fecund females pregnant during the year is 0.2194 ± 0.0199 for urban, 0.3166 ± 0.0208 for flour buffer depot, 0.4060 ± 0.0227 for rick and 0.2653 ± 0.0193 for cold-store mice. Each of these figures can be interpreted as the average rate of litter production per 14.5 days, the period of gestation during which pregnancy is visible to the naked eye. To obtain the yearly rates, together with their standard errors, each of the above is multiplied by the factor $365/14.5 = 25.17$. The productivity figures thus obtained are as follows:

urban	5.52 ± 0.50	ricks	10.22 ± 0.57
flour depots	7.97 ± 0.52	cold stores	6.68 ± 0.49

The theoretical maximum number of litters which may be produced by one adult female mouse during a year, assuming that fertilization takes place at each post-partum oestrus, can be calculated on the assumption that the gestation period is prolonged from 19.5 to 27.5 days, by the addition of 8 days which may be taken as the average period by which the gestation period of suckling mice is prolonged (see p. 250). This gives the maximum number of litters per pregnant female per annum as 13-14.

It will be seen that the actual annual productivity figure for the rick mice is approaching this theoretical maximum.

B. *Embryo rates*

For a fuller knowledge of fertility some study of embryo rates must be undertaken. If all embryos were born it is clear that embryo rates could be used to calculate fertility with considerable accuracy, but as indicated above, in the section dealing with size of litter, Brambell finds for certain laboratory-bred mice a foetal elimination of approximately one-third. Actually in the present investigation 59 complete litters are recorded from the ricks, with an average of 5.83 nestlings per litter (table 1), which is not very different from the average of 5.37 embryos for 227 pregnant rick females (table 16, p. 277). It would thus appear that embryo counts

as carried out in the present investigation, in which only those embryos have been counted which are visible by the naked eye and deducting the 3% (37 out of 1257) which are evidently undergoing resorption, may be taken as being substantially equivalent to fertility rates. A deduction of a further 3% for estimated further resorption as yet undisclosed may be made from such embryo counts to give a corrected figure for the number of young expected to be born. In the present instance the application of the correction reduces the expectation to 5.21.

(a) *Embryo rates, weight and environment*

As in dealing with the pregnancy rates, weight is, in the absence of knowledge of age, taken as the basis of classification. In studying pregnancy rates the percentage pregnancy was found, however, to be influenced by weight, and a correction had to be introduced before valid comparisons could be made between different samples. It is desirable to find out whether the number of embryos in the uterus is also related to the weight of the parent (tables 13, 14). It might appear on first sight, on reference to the means given in table 13, that in spite of some irregularity there is some relation between the mean number of embryos and the weight of the parent, for buffer depot, urban and rick mice. This was tested by the method of the analysis of variance, as also the relation between the environment and the number of embryos for the same environments, and it was found that neither weight nor habitat is statistically significant. Similarly, it was found among the cold-store mice (table 14), by use of the same test, that there is no association between the number of embryos and the weight of the parent.

It has already been seen in the section dealing with pregnancy that the cold-store mouse reaches on the average a greater weight before breeding than is the case for mice from other environments. Tables 13 and 14 (figures 3-6) show that the mean number of embryos per pregnant female is greater in cold-store mice than in those from other environments. The difference between the mean number of embryos in the cold stores and that of the other environments, divided by its standard error, gives the statistically significant value of $t = 3.5$.

To gain some idea of the frequency distribution and average number of embryos among pregnant females of *Mus musculus* generally, those from the cold stores are best omitted, the environment hardly ranking as a 'natural' one. The best indication is obtained by summing the data for the other three environments as in the last column of table 13. The mean value is 5.60 ± 0.08 .

(b) *Seasonal differences*

From the figures for the average number of embryos per pregnant female which are given in table 15, it does not appear as though there was any consistent increase or decrease in fertility at any particular time of the year. The samples are too small to be tested statistically by the analysis of variance, but the differences which do occur may well be explained by the small number in the sample and the wide range of 2-13 in the number of embryos which a pregnant female may bear.

(c) *Annual productivity*

(i) *In terms of the number of embryos.* On p. 272 the productivity of mice from the various environments was stated in terms of litter production (without prejudice to size of litter) to be for urban habitats 5.52 litters per annum, buffer depots 7.97, ricks 10.22 and cold stores 8.68. Multiplying each of the first three productivity figures by the average number of embryos per pregnant female for the three environments taken together (table 13) the embryo productivity is as follows: urban 30.91, buffer depots 44.63, ricks 57.23. For the exceptional environment of the cold stores the equivalent figure is 42.55.

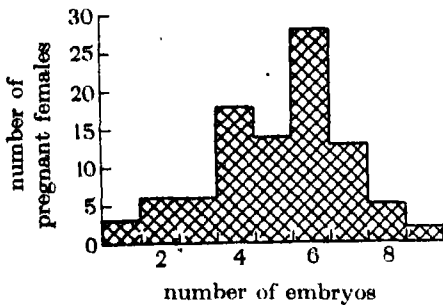


FIGURE 3. Distribution of embryos of 95 pregnant females of *Mus musculus* from urban habitats.

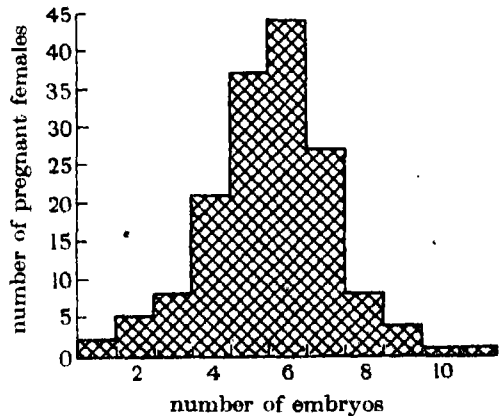


FIGURE 4. Distribution of embryos of 158 pregnant females of *Mus musculus* from flour buffer depots.

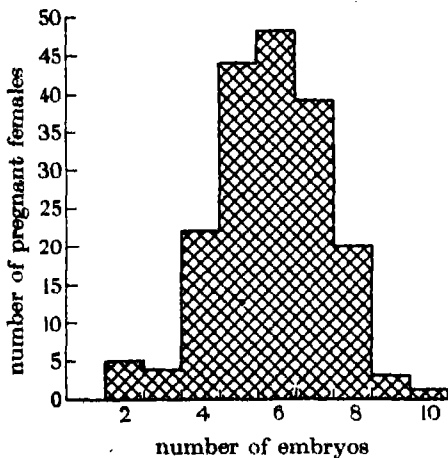


FIGURE 5. Distribution of embryos of 186 pregnant females of *Mus musculus* from corn ricks.

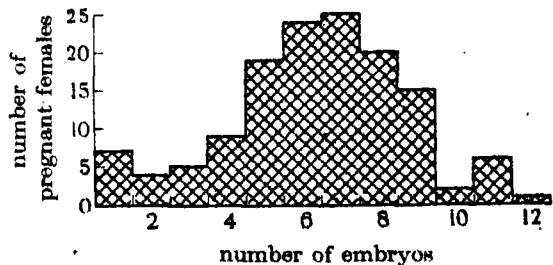


FIGURE 6. Distribution of embryos of 137 pregnant females of *Mus musculus* from cold stores.

TABLE 14. DISTRIBUTION OF THE NUMBERS OF EMBRYOS PER PREGNANT FEMALE IN RELATION TO WEIGHT FROM THE COLD STORES

number of embryos	weight group of pregnant female (g.)					grand total
	12.5-	17.5-	22.5-	27.5-	32.5-	
1	2	2	2	1	—	7
2	—	—	1	3	—	4
3	—	2	—	2	1	5
4	1	1	4	2	1	9
5	3	6	5	3	2	19
6	1	9	6	5	3	24
7	2	6	12	2	3	25
8	—	4	11	5	—	20
9	1	3	7	4	—	15
10	—	—	1	1	—	2
11	—	1	2	3	—	6
12	—	—	1	—	—	1
total	10	34	52	31	10	137
mean	5.00	6.12	6.92	6.42	5.60	6.37
S.E. \pm	0.7616	0.3620	0.3123	0.5055	0.4171	0.2036

TABLE 15. THE TOTAL NUMBER OF PREGNANT FEMALES (N.), EMBRYOS (E.) AND AVERAGE NUMBER OF EMBRYOS (AV.) FOR THE FOUR HABITATS, FROM 8 JAN. 1942 TO 7 JAN. 1943

date	buffer depots			urban			ricks			cold stores		
	N.	E.	av.	N.	E.	av.	N.	E.	av.	N.	E.	av.
8 Jan.-4 Feb.	9	48	5.33	2	12	6.00	31	162	5.23	14	106	7.57
5 Feb.-4 Mar.	17	93	5.47	2	12	6.00	26	140	5.60	5	22	4.40
5 Mar.-1 Apr.	4	19	4.75	10	47	4.70	16	100	6.25	16	102	6.38
2 Apr.-29 Apr.	39	216	5.54	3	17	5.67	9	56	6.22	18	113	6.28
30 Apr.-27 May	28	145	5.18	12	71	5.92	2	13	6.50	8	39	4.88
28 May-24 June	5	33	6.60	6	27	4.50	3	16	5.33	4	20	5.00
25 June-22 July	2	13	6.50	10	48	4.80	26	134	5.15	3	24	8.00
23 July-19 Aug.	1	5	5.00	2	9	4.50	—	—	—	4	31	7.75
20 Aug.-16 Sept.	15	81	5.40	5	23	4.60	—	—	—	18	107	5.94
17 Sept.-14 Oct.	25	125	5.00	5	28	5.60	19	103	5.42	2	11	5.50
15 Oct.-11 Nov.	8	64	8.00	11	57	5.18	1	9	9.00	10	50	5.00
12 Nov.-9 Dec.	5	37	7.40	14	77	5.50	—	—	—	4	22	5.50
10 Dec.-7 Jan.	—	—	—	13	58	4.46	59	368	6.24	33	219	6.64

(ii) *In terms of the number of daughters born.* The percentage number of males to females among nestlings is given in table 5. Using these figures it is possible to calculate the number of daughters produced per pregnant female per annum by multiplying the above embryo productivity figures by their respective ratios for each environment. The results are as follows: urban 16.22, buffer depots 24.79, ricks 31.51 and cold stores 23.15.

(d) *The distribution of the embryos in the uterus*

In 377 pregnant females the distribution of the embryos between the right and left horns of the uterus was noted in order to ascertain whether it is random. Table 16 shows the results obtained. In one instance a total absorption of all the embryos in both horns was noted.

It is at once evident that there is an unequal distribution of embryos between the two horns, and that this is in favour of the right horn. In each habitat considered separately the total number of embryos in the right is greater than in the left, giving a grand total of 2094 embryos, of which 1107 are in the right horn and only 987 in the left.

TABLE 16. DISTRIBUTION OF EMBRYOS IN THE RIGHT
AND LEFT HORNS OF THE UTERUS

	normal embryos				number pregnant females	resorbing embryos		
	R.	L.	total	av.		R.	L.	total
buffer depots	156	137	293	5.86	50	3	3	6
urban	138	102	240	5.11	47	4	7	11
cold stores	181	160	341	6.43	53	1	3	4
ricks	632	588	1220	5.37	227	16	21	37
total	1107	987	2094	5.55	377	24	34	58

This predominance of embryos in the right horn is statistically significant, the value of P obtained from χ^2 between the totals, with $n = 1$, being approximately 0.01. A much more sensitive test, however, is to compare the actual distribution in the right and left horns with the expected binomial distribution $(\frac{1}{2} + \frac{1}{2})^n$, n being the number of embryos. Testing this distribution by the χ^2 obtained by comparing the observed with the binomial for each frequency (grouping the data when the expected frequency is below 5) gives $\chi^2 = 44.0239$, which with $n = 20$ gives a value of P less than 0.01 which is highly significant.

It is difficult to suggest why there should be this predominance of embryos on the right side. It might be due either to an ovarian or uterine function, i.e. an uneven distribution of follicles between the two ovaries or an uneven mortality in the uterus. As the number of corpora lutea in the ovaries was not counted, comparisons between the number of corpora lutea and the number of embryos cannot be made. Also it is not possible to ascertain the amount of transference which may take place from one side to the other, via the junction of the two horns. From the data obtained (table 16) the suggestion arises that embryos may be absorbed more often in the left horn than in the right, affording some slight evidence in favour of differential uterine mortality as the explanation.

Biased distribution in favour of the right horn does not appear to have been noted in laboratory bred mice or in other rodents, where the distribution is entirely random. Thus Danforth & de Aberle (1928) examined the distribution of the

foetuses of 500 pregnant laboratory-bred mice and found 1579 in the left as compared with 1594 in the right, and Parkes (1924*b*) found the total distribution of the embryos of 13 pregnant female laboratory-bred mice to be 57 in the right horn, and 59 in the left.

The most common distribution for wild *Mus musculus* was found to be equality, i.e. 3 in the right and 3 in the left horn, the modes of the distribution for the two horns separately also being 3 left and 3 right. Danforth & de Aberle (1928) found the most common association of foetuses in 500 uteri of laboratory-bred mice to be 3 left with 4 in the right, with separate distribution modes of 3 in the left and 3-4 in the right. They also found the modal number of foetuses to a litter to be 7, against 6 for wild *Mus musculus* in the present investigation.

10. DISCUSSION

Mus musculus differ from all other British wild rodents in reproducing at a very even rate throughout the year. The typical breeding season for British wild rodents shows a cessation of breeding during the winter months from about October to February, e.g. *Microtus agrestis* has a breeding season which lasts from February or March to September or October (Baker & Ranson 1933). In the brown rat, *Rattus norvegicus*, breeding is very low from November to February except for those which live in the corn-ricks where some 28% of the adults were pregnant against 3% for the non-rick females, the peak period for both environments being in April (Venables & Leslie 1942):

The low rate of pregnancy found among the urban mice as compared with that for other environments is probably associated with the comparative instability of their habitat, i.e. the greater amount of disturbance to which they are subjected, and their more scanty diet.

In the flour depots the diet consisted of nothing but white flour, which one might expect to be a very unsuitable diet for optimum reproduction. It might be supposed that the tendency to eat their young which occurs in mice may be more pronounced among the mice in this environment on account of their apparently deficient diet. The pregnancy rate is, however, higher than either that for the cold store or urban environments. Here again the mice suffer little from disturbance either from man or natural predators.

It does appear though that the complete diet of flour is the cause of a skin disease first noticed by Mr Charles Elton among some of the mice particularly the males. This is characterized by a very thin pelage, bald patches and crumpled and torn ears. It was most prevalent among the mice from one particular flour depot, but its occasional occurrence among those from others supports the view that it is due to the diet and not a weakness peculiar to a particular strain. The majority of the depot mice are unaffected and appear to be quite normal and healthy.

The rick is a temporary environment but while standing provides a stable and secure habitat, with no great extreme in temperature and an unlimited food supply

of grain. The mice living in it are comparatively free from predators such as weasels, stoats, kestrels, owls, etc., and are afforded very favourable conditions for rapid increase. The largest complete population of mice was 2368, counted from a nine months old wheat rick and equivalent to a density of 0.43 per cu.ft. (15.0 per cu.m.). The following sample though small represents a fairly complete population obtained from an oat rick about four months old and is typical of several of similar age which have been examined.

TABLE 17. A FAIRLY COMPLETE POPULATION OF A 4 MONTH OLD OAT RICK

weight group g.	females					males		
	number	non- fecund	fecund	pregnant	embryos	number	non- fecund	fecund
0.0-	4	4	—	—	—	2	2	—
2.5-	15	15	—	—	—	13	13	—
5.0-	8	8	—	—	—	7	7	—
7.5-	3	2	1	—	—	7	7	—
10.0-	6	—	6	—	—	9	8	1
12.5-	7	—	7	4	22	8	1	7
15.0-	12	—	12	6	40	12	—	12
17.5-	8	—	8	6	37	3	—	3
20.0-	1	—	1	—	—	—	—	—
total	64	29	35	16	99	61	38	23

45.71 % fecund females pregnant; 37.70 % males fecund; average number embryos 6.19.

The potential rate of increase of mice in a corn rick is very formidable so that, as has been pointed out by Venables & Leslie (1942, p. 66), they may cause more damage than rats. One adult mouse eats about 3.0 g. of corn a day, so that 2000 in a rick would be eating approximately one stone (6 kg.) a day. Apart from this total consumption of grain a large quantity is wasted through being nibbled.

The conditions of the cold stores are of particular interest not only on account of the very low temperature of anything from -5 to 15°F (-21 to -10°C) below freezing-point and the meat diet, but also on account of the practically constant nature of these features.

Parkes (1924b) found that without artificially maintaining an optimum temperature of 55 – 65°F (13 – 18°C), his albino mice failed to breed in the months of November–February, whereas Parkes & Brambell (1928) found that kept at about 0°C with abundance of food they had regular oestrous cycles and became pregnant when copulation was allowed.

In connexion with a protein diet Cunningham & Hopkirk (1935) found that sterility was induced in male rats fed on an excessively high protein diet of 65–82 % animal protein, i.e. dried blood, casein and meat meal, as also was found to be the case by Reader & Drummond (1925). Female rats, however, remained fertile throughout the year. The young were healthy and normal though their size and weight were less than for stock rats, but the parents were unable to rear their young

on account of their deficiency in vitamin B. No indication of this sterility has been found among the cold-store mice.

It is noteworthy that the pregnancy rate in the cold-store mice remains relatively high. This may be accounted for by the balance between the low temperature and the entirely meat diet upon which they live, as well as by the comparative lack of disturbance as compared with the mice from domestic habitats. Within their nests, made from hessian or often among the feathers of fowl, they will naturally produce a warmer microclimate in which to rear their young. Nevertheless they come out into the cold store in search of food.

As has already been pointed out, the mice from the cold stores attain a much greater size and weight than those from any other environment, which would appear to be related to the temperature.

The effect of temperature upon growth of mice has been studied by various workers. Mills (1942) gives a description of results obtained by W. Calvin in the Laboratories for Experimental Medicine at Cincinnati. Using air conditioned rooms, one simulating continuous tropical moist heat, 91° F (33° C) and 60% relative humidity, and another temperate coolness, 65° F (18° C) (relative humidity not mentioned), he found that young white mice put into the cold room continued to grow and develop at a rapid rate, but those in the hot room suffered at once from marked and sustained depression, their comparative weights at the age of 21 days being about 29 and 21 g. respectively. This greater development of the mice at the lower temperature occurs under an optimal diet, though the nature of the diet is not specified. They are not so long lived as those at the higher temperature but on the other hand have a greater capacity to resist disease.

Przibram's work (1925) supports the rules of Allen and Bergmann who concluded that in warm-blooded species the exposed portions of the body decrease, while the size of the body increases with decreasing environmental temperature. He also concludes that such modifications are not permanently inherited.

Mice in cold stores are practically free from natural predators. Occasionally cats become acclimatized to living in the stores, but this is not usual. The meat usually remains in stacks for some time and so the mice are left undisturbed.

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Filtration of droplets in the nose of the rabbit

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[Plates 1–3]

Airborne droplets or nearly spherical particles down to a diameter of about 7μ will be filtered out in the nose of a rabbit. Those from 1.5μ down to about 0.005μ , which corresponds to a single molecule of molecular weight of the order of 10,000, will pass freely into the lungs. Molecules of molecular weight below about 500 will be effectively filtered out in the nose unless accumulation in the tissues near the surface becomes a controlling factor. The filtering unit, which also provides most of the resistance to air flow, comprises the maxillary turbinates.

1. INTRODUCTION

Research on dangers caused by the inhalation of dust is often carried out upon animals; in order to apply the results towards assessing risks to human subjects, it is necessary to bear in mind such differences in the respiratory organs and processes as may exist and to make due allowances. This paper describes a study of nasal filtration in the rabbit which was undertaken with such an end in view. When particles suspended in the atmosphere are inhaled some will get trapped in the nose; the work was directed to finding out the filtration efficiency of the nose, as a function of particle size, with specified steady air flows. From the results, the extent of filtration, under natural conditions, can be gauged and a basis provided for discussion of the biochemical and physiological processes which follow the deposition of particles. Droplets were used in the experiments for technical convenience, but the results are applicable to solid particles providing their shapes do not deviate too much from the spherical.

Very small drops, and vapour molecules, reach the walls of the respiratory tract by diffusion, and the rate of transfer of reagent to the tissues of the animal is governed by two interdependent factors. The external factor is the rate of diffusion to the surface; the internal factor is the rate of absorption or of chemical reaction which, when slow, may set up a diminished concentration gradient in the external diffusion zone and so retard the net transfer. Drops about 0.5μ in diameter diffuse at a rate which is several hundred thousand times slower than that of a typical organic vapour; for larger drops deposition by diffusion is quite negligible.

A drop is constrained to follow the streamlines of flow of the suspending atmosphere by a force due to viscosity which is proportional to its surface area. On the other hand, its inertia tends to make it continue to move in a constant direction and so cut across the flow lines where these bend; when this happens it has a chance of coming into contact with the bounding surfaces. Thus the chance of deposition, dependent upon the inertia force overcoming the viscous, increases with drop diameter. It is a function of external and geometric considerations and is not affected by saturation phenomena as in the case of vapours.

2. METHOD OF EXPERIMENT

Animals used in the filtration experiments were prepared and handled by Dr E. Boyland who writes: 'Rabbits weighing between 2.0 and 3.2 kg. were anaesthetized by intraperitoneal injection of nembutal (0.5 c.c./kg. body weight). An incision was made into the neck, and a cannula bent to a right angle inserted into the trachea and tied so that the arm in the trachea communicated with the lungs. The trachea was cut and a second cannula in the form of a T inserted to connect with the nose. The muzzle of the animal was then introduced into the apparatus through a hole in a rubber diaphragm which formed a fairly airtight connexion without pressing upon the nostrils. The nose was next tested to see that it was free from obstruction and that the side branch of the T cannula connected to a sampling apparatus. The other arm of the T was closed by a screw clip. In some experiments the mouth was sewn up, but this had no effect upon the results, and in no case was there any indication of the cloud having been sucked through the mouth.'

A cloud of droplets of Apiezon oil B covering the correct range of sizes was passed down a tube into the side of which the nose of the animal was projecting. Part of the cloud was drawn through the nose and led at a constant rate of flow through cells where sedimentation samples could be trapped without interrupting the flow. Immediately after taking these samples portions of the original cloud were similarly trapped.

After sedimentation had taken place microscopic counts were made from which comparisons of the original and passing clouds could be drawn. Experiments were carried out on a number of different rabbits using a range of air flows. The resistance to flow through the nose was also measured.

The success of this type of experiment depends on using a cloud of droplets whose sizes just cover the range in which penetration changes from 100 % to nearly zero. The droplet spectrum of the spray employed could be adjusted by varying the air flow, and this was set, as described below, after a preliminary trial.

3. DETAILS OF THE APPARATUS

The apparatus consisted of three essential units illustrated in figures 1-3.

Apiezon oil B was fed at a constant rate (0.024 c.c./min.) to a spray, where it was atomized by air emerging from an outer tube coaxial with the liquid capillary. This had 0.47 mm. internal and 1.0 mm. external diameters. The coaxial air tube was 1.44 mm. internal diameter at the orifice. The air pressure was kept at 25 cm. mercury by a needle valve and manometer. The cloud from the spray (13 l./min.) was diluted with 20 l./min. of air in a bulb about 9 cm. diameter, the extra air being controlled by a flowmeter and needle valve and led into the bulb tangentially to ensure good mixing and the removal of the coarser drops. The diluted cloud passed down 50 cm. of glass tube 4 cm. in diameter to the sampling region.

The theoretical concentration of cloud was thus 0.024 c.c. in 42 l. or 0.57 c.c./m.³. The mean measured concentration at the sampling region was 0.083 c.c./m.³ (see below) or 14.6 % of the theoretical. The balance of the oil was collected from time to time from the bottom of the mixing bulb upon the walls of which the larger drops of the cloud impacted.

At the sampling region a short side-arm was fixed and covered with a thin rubber diaphragm, into a hole in which the muzzle of the animal could be inserted; on the opposite side was a tube connected to an inclined manometer. In the bottom a 1.5 cm. diameter tube led by a short, wide rubber tube to one manifold of the sedimentation apparatus, and through this the original cloud was drawn. This tube is marked *A* in figure*1.

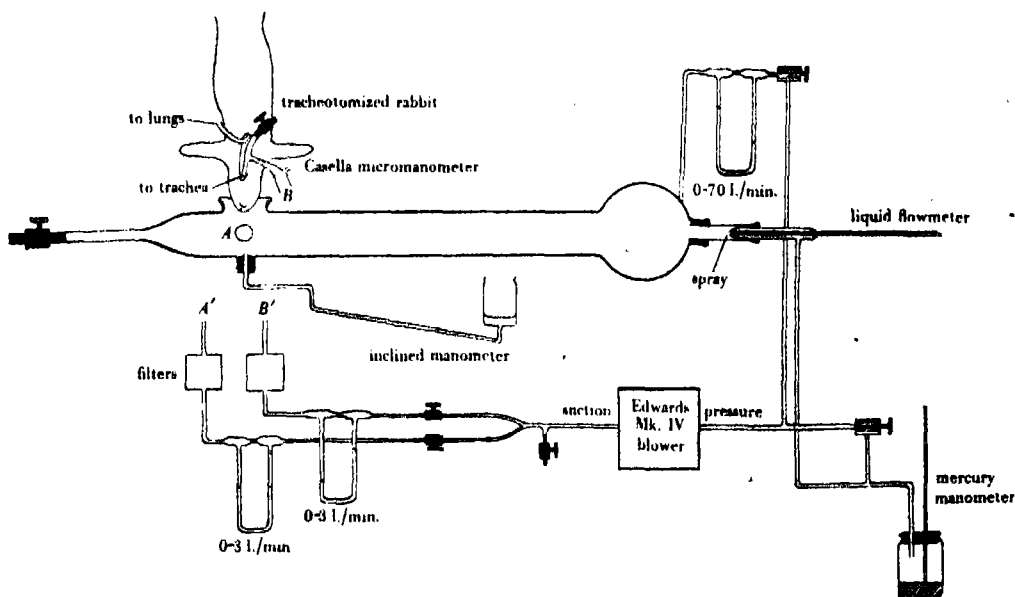


FIGURE 1. Apparatus for measuring penetration of drops through the nose of a rabbit.

The trachea of the rabbit was connected to a T-piece of suitable diameter; when an experiment was commenced the straight, open end of this was closed by a screw clip. The side arm opened up into a 1.5 cm. diameter tube which led directly through a short, wide rubber tube connexion to the other manifold of the sedimentation cells. This lead is marked *B*. By means of a Casella micromanometer the pressure at *B* could be held equal to atmospheric when measuring resistances, or slightly above it when taking the cell samples.

The outlets from the sedimentation cells connected at *A'* and *B'* through filters, flowmeters and screw cocks to the suction side of an Edwards Mk. IV blower which fed air, from the pressure side, into the system as described above. This particular circuit arrangement was used, since, by screwing up the cock on the outlet of the main tube below the sampling region, a small positive pressure could be built up

inside, which was equal in magnitude to the pressure drop across the nose of the animal. In this way it was possible to maintain atmospheric pressure, or in practice a slight excess, inside the sedimentation cells. If a more normal circuit, involving suction only, had been employed, the pressure in the cells of the passing cloud would have been below atmospheric with the result that air would have leaked in. It is also probable that less difficulty is experienced with stoppages in the nasal passages. With this procedure it was not necessary for the slides of the cells to be a perfect fit; slight leakage here could cause no trouble. It was found convenient to use Spencer Wells forceps on the rubber inlet and outlet tubes of the cells so that once the samples were obtained they could be quickly isolated. The outlet was clipped before the inlet so that any leakage would be outwards and, hence, unimportant.

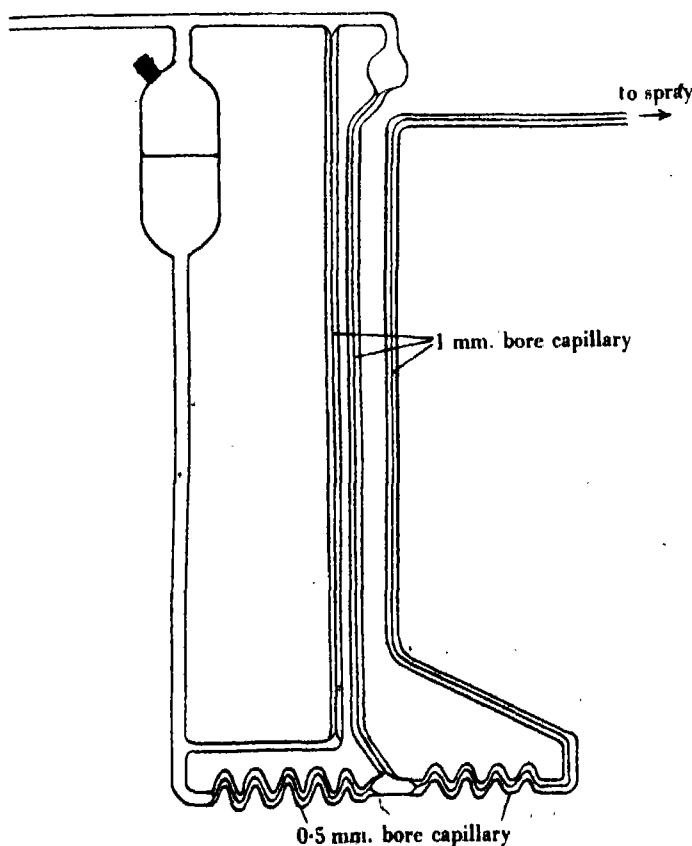


FIGURE 2. Liquid flowmeter.

The liquid flowmeter consisted of a reservoir from which oil was driven to the spray by air pressure through two helices of capillary tube in series. The pressure drop across the first of these was measured by a pair of capillary manometric tubes which were calibrated so that the difference in head of liquid across the helix was related to

the flow. The purpose of the second helix was to provide a resistance to prevent the suction of the spray from taking charge. Control of flow was obtained by variable air pressure indicated on a mercury manometer (0-10 cm.). The capillary helices were immersed in a water-bath thermostated at 25° C. The flow of liquid to the spray, as well as the air, was kept constant in this manner, so that a reproducible cloud was obtained.

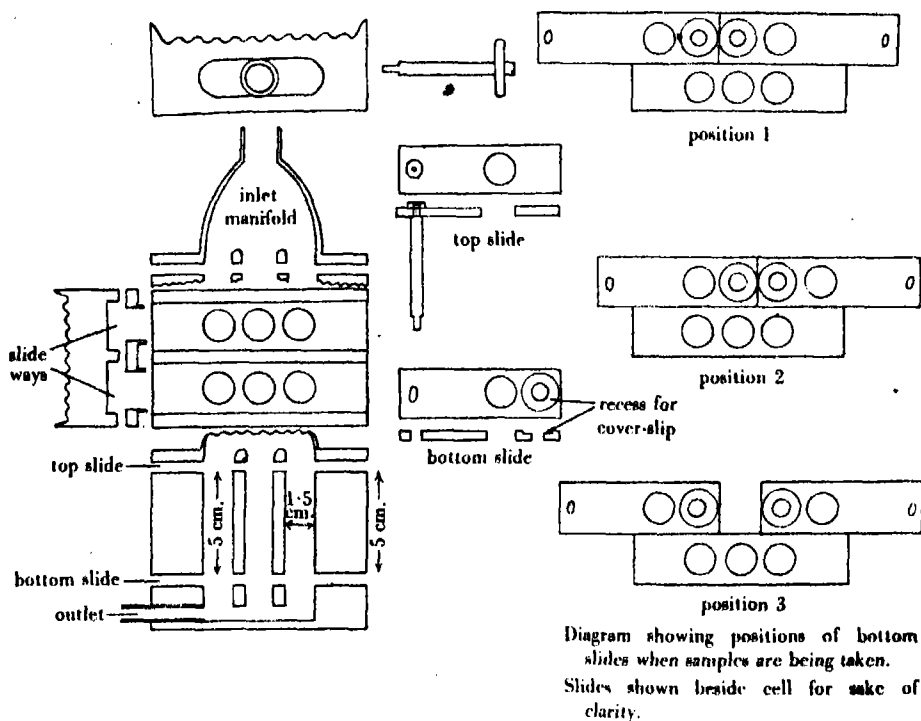


FIGURE 3. Sedimentation apparatus for original and passing clouds.

The sedimentation apparatus comprises a block of brass containing six parallel cylindrical cells 5 cm. deep by 1.5 cm. diameter arranged in two banks of three. One bank is for the original cloud and one is for the passing, each being one of identical but independent systems. The cloud enters through a manifold which communicates with all three cells but is only able to enter one at a time, the selection being controlled by a pair of opposed top slides. A rod on each of these engages with one of a pair of bottom slides which regulate the exit of the cloud into an exhaust duct common to the three cells. In a manner which is shown diagrammatically in figure 3 two sedimenting samples of cloud can be trapped in the outer cells, in sequence, without disturbing the total flow which finally passes through the centre cell.

Two hours' sedimentation suffices for drops exceeding 0.5μ diameter or 7 hr. for those above 0.2μ .

The apparatus is suitable for rates of flow and drop diameters up to maxima governed by impaction and sedimentation losses in the sampling line. This was

kept as short and wide as possible. As far as the cells are concerned it can be stated definitely that no appreciable errors arise due to losses for flows up to 10 l./min. and drops up to 20μ diameter. This is equivalent to a mean velocity of 92 cm./sec. through the cells, which is large compared with the terminal velocity of 20μ drops (1.2 cm./sec.). At this velocity 20μ drops have only enough kinetic energy to traverse 0.1 cm. of still air so that wall losses will be slight. In addition, turbulence in the stream is unlikely to be serious.

4. EXPERIMENTAL DETAILS

The Apiezon oil B employed had the following properties: $\mu_p^{20} = 1.545$, $\rho_{25} = 0.858$ g./cm.³, $\eta_{25} = 0.541$ poise, vapour pressure about 10^{-6} mm. mercury at 20° C.

At the commencement of work with this apparatus a cloud of drops of dibutyl phthalate was used which has a vapour pressure of 3×10^{-5} mm. at 20° C and boils at 340° C. It was found that drops below 5μ diameter were subject to loss by evaporation, and after attempts had been made to combat this by saturating all air streams with vapour it was found more satisfactory to use Apiezon oil which gave no trouble. The lifetimes of isolated drops of dibutyl phthalate are: 0.2μ diameter, 47 sec. and 2.0μ diameter, 3050 sec.

Drops were allowed to sediment on to glass cover-slips. At the conclusion of an experiment the slip was removed from the bottom slide, inverted and laid over a cavity ground in a special type of microscope slide which it just covered. A seal was then made by running a very small amount of Apiezon oil which spread by capillarity right round the circumference of the slip. In this way a firm seal was obtained which prevented evaporation by saturating the space around the drops with oil vapour. Correct treatment of the glass surfaces to ensure reproducible results was crucial to the success of the experiments. Attempts were first made to get absolutely clean glass surfaces by storage in chromic acid solution, washing and then flaming with a bunsen; but this was quite hopeless, as the drops spread irregularly to a considerable extent and could not be sharply focused. Slips rinsed and polished after storage in chromic acid were better but showed the curious effect illustrated in figure 4, plate 1. Surrounding the larger drops on the slip was a relatively clear space from which the background of more numerous small drops had been removed. This phenomenon was not particularly consistent, being much worse on some occasions than others, and it is thought to have been due to a very thin film of oil creeping over the surface from the larger drops. When this film encountered small drops they probably spread into it and disappeared. It was found that the following technique gave the best results with freedom from this defect. The slips were stored in chromic acid solution and then removed, rinsed, and dropped into 0.1 % aqueous solution of the wetting agent Aerosol O.T. After keeping them in this for an hour or more the slips were dried, lightly polished and inserted in the apparatus. It was found advantageous to reject cover-slips of poor optical quality

by spreading them out on a table and examining the reflexion of a window in both surfaces.

By this method the spread of drops was consistent for sizes down to about 0.5μ diameter, the criterion being reasonable agreement between counts on each of the pairs of cover-slips exposed in the slides. Smaller drops, however, were not remarkably consistent, and it is probable that a different technique would be best for passing clouds which had a maximum drop size of this order.

After sizing and counting the drops allowance was made for their spread on glass. The drops formed plano-convex lenses, the focal length of which was measured with a microscope having a graduated scale on the fine focusing adjustment. Hence, knowing the refractive index of the oil, the angle of contact could be deduced and the volume of the lens worked out. It was assumed that the surface was spherical. In order to facilitate the large number of calculations needed a special chart was prepared. On each slide a number of drops was dealt with covering the range of sizes required and one or more factors determined to reduce lens diameter to equivalent spherical drop diameter. The smallest diameter of lens that it was possible to measure in this way was 4μ . The reduction factor was always between 0.42 and 0.52.

5. COMPUTING TECHNIQUE

From a successful experiment two slips, *A* and *B*, from the original cloud cells and two from the passing cloud cells were available. Counts on each were made using 4 and 12 mm. objectives and a 17 times eyepiece fitted with a Patterson-Cawood (1936) graticule. With the 4 mm., 60 fields, defined by one-third of the long side of the graticule by the short side, were counted sizing only drops greater than the 2 circle. 60 fields were then counted, the long side by the short side, sizing drops greater than the 4 circle. Finally, using the 12 mm. objective, 60 fields were counted, long side by short side, sizing drops greater than the 4 circle. By adjusting the tube length the following relations were maintained:

size in μ	24	18	15	12	9.6	7.2	6	4.8	3.8	2.9	1.9
4 mm. objective	—	37	—	25	20	15	12.5	10	8	6	4
12 mm. objective	20	15	12.5	10	8	6	—	4	—	—	—

} circles

In this manner representative counts of the sparse larger drops were obtained. The counts were lined up, in tabular form, with a column for each set of 60 fields and a line for each size range. The blank spaces were then filled in by averaging the existing counts in the same line, each being weighted according to the size of field. The numbers in each line were then assumed to give the total 'counted' in each size range in 455 effective fields, long side by short side (4 mm.), or $120 \times 53.3\mu^2$. The total was obtained as follows:

$$[60 \times 1/3(LS \times SS) + 60 \times (LS \times SS)] \text{ 4 mm. } + [60 \times (LS \times SS)] \text{ 12 mm.}$$

$$= [80 \times (LS \times SS)] \text{ 4 mm. } + \left[\frac{300}{120} \times \frac{133}{53.3} \times 60 \times (LS \times SS) \right] \text{ 4 mm.}$$

$$= (80 + 60 \times 6.25) (LS \times SS) \text{ 4 mm. } = 455 \text{ fields } (LS \times SS) \text{ 4 mm.}$$

TABLE 2. EXPERIMENTAL DATA

experiment number	cloud	size ranges μ (above the 2 circle)										number per field $120 \times 53.3\mu^2$ total counted	mean number per field	mean concentration c.c./m. ³	diameters μ			
		number counted													50 % number	mean mass	50 % mass	
68 A	original	0.89	1.36	1.76	2.26	2.9	3.4	4.5	5.6	7.0	8.5	11.0	13.55	0.077	0.65	1.44	3.2	10.6
68 B	original	4366	906	396	153	143	117	52	10.92	12.12	4.86	2.42	6157	15.73				
68 A	original	0.84	1.28	1.67	2.11	2.64	3.16	4.22	5.28	6.6	8.0	10.6	8230					
68 A	passing	4904	1748	842	935	188	136	48	6.06	4.86	4.86	3.41	10.52					
68 A	passing	3228	942	274	136	164	47	7	—	—	—	—	4796	8.11	0.033	0.88	1.39	2.28
68 B	passing	0.84	1.28	1.67	2.11	2.64	3.16	4.22	5.28	6.6	8.0	10.6	5.7					
68 B	passing	893	668	454	250	204	91	25	1.21	—	—	—	2586					
68 A	original	0.83	1.28	1.67	2.11	2.64	3.16	4.22	5.3	6.6	7.9	10.6	15.3	0.047	0.64	1.23	2.8	10.6
68 A	original	5000	1151	415	148	118	84	35	9.8	2.42	1.19	1.19	6966	15.3				
68 B	original	0.83	1.28	1.67	2.11	2.64	3.16	4.22	5.3	6.6	7.9	10.6	8.36					
68 A	passing	2768	664	210	51	45	31	4.85	2	—	—	—	3806	8.43	0.014	0.64	1.01	1.9
68 B	passing	0.83	1.28	1.67	2.11	2.64	3.16	4.22	5.3	6.6	7.9	10.6	8.5					
68 B	passing	2820	743	159	51	5	32	4.85	—	—	—	—	3860					
70 A	original	0.89	1.36	1.79	2.26	2.82	3.4	4.5	5.7	7.0	8.5	11.3	12.5					
70 B	original	3544	1363	364	165	131	110	47	21	7.28	6.0	1.21	5899	12.6	0.066	0.80	1.48	3.2
70 B	original	0.8	1.2	1.6	2.0	2.5	3.0	4.0	5.0	6.3	7.5	10.0	12.7					
70 A	passing	3736	1116	420	165	160	109	46	15	8.5	4.0	1.21	5781					
70 A	passing	0.91	1.35	1.81	2.26	2.82	3.4	4.5	5.7	7.0	8.5	11.3	9.6	0.035	0.80	1.30	2.3	7.0
70 B	passing	3009	764	306	148	63	55	18	2.42	—	—	—	4385	9.62				
70 B	passing	0.91	1.35	1.81	2.26	2.82	3.4	4.5	5.7	7.0	8.5	11.3	9.65					
70 B	passing	2643	1034	335	160	131	61	29	3.64	1.21	—	—	4398					
71 A	original	0.85	1.3	1.7	2.16	2.7	2.2	4.3	5.4	6.7	8.1	10.8	11.9	0.074	0.80	1.55	3.1	11.0
71 B	original	3497	1043	386	170	165	114	43	12	3.66	4.0	2.43	5440	12.0				
71 B	original	0.88	1.3	1.7	2.16	2.7	2.2	4.3	5.4	6.7	8.1	10.8	12.1					
71 A	passing	3181	840	636	233	233	175	87	21	1.21	4.0	—	5484					
71 A	passing	0.89	1.36	1.79	2.26	2.82	3.4	4.5	5.7	7.0	8.5	10.5	10.5					
71 B	passing	2662	1326	278	182	158	90	47	9	6.1	3	—	4791	10.8	0.067	0.85	1.56	3.0
71 B	passing	0.89	1.36	1.79	2.26	2.82	3.4	4.5	5.7	7.0	8.5	10.5	11.1					
71 B	passing	2778	1209	415	245	170	110	59	12	2.42	2.42	—	5063					
72 A	original	0.90	1.51	1.98	2.5	3.1	3.74	5.0	6.3	7.8	9.4	—	13.5					
72 B	original	4903	743	165	119	58	40	36	12.3	10.92	2.0	—	6139	12.06	0.069	0.80	1.66	4.0
72 B	original	0.99	1.51	1.98	2.5	3.1	3.74	5.0	6.3	7.8	9.4	12.5	10.6					
72 A	passing	3459	757	194	125	125	95	37	14	9.71	1	—	4818					
72 A	passing	0.99	1.51	1.98	2.5	3.1	3.74	5.0	6.3	7.8	9.4	11.4	11.4					
72 B	passing	3774	883	240	159	62	33	2.43	1.21	—	—	—	5165	10.9	0.032	0.80	1.21	2.0
72 B	passing	0.99	1.51	1.98	2.5	3.1	3.74	5.0	6.3	7.8	9.4	10.4	10.4					
72 B	passing	3457	708	204	131	63	36.2	4.85	—	—	—	—	4604					

experiment number	cloud	size ranges μ (above the 2 circle)										number per field 120 \times 53.3 μ^2 total counted	mean number per field	mean concentration c.c.m. ³	diameters μ			
		number counted													50 % number	mean mass	50 % mass	max.
73 A	original	0.99	1.51	1.98	2.5	3.1	3.74	5.0	6.3	7.8	9.4	12.5	13.7					
		4051	1056	425	279	171	157	70	24	5	5.86	2.43	62.66					
73 B	original	0.99	1.51	1.98	2.5	3.1	3.74	5.0	6.3	7.8	9.4		14.8			1.71	3.9	12.0
		4685	1129	403	165	131	155	47	13	5.86	5.43		67.39					
73 A	passing	0.99	1.51	1.98	2.5	3.1	3.74	5.0	6.3				11.5					
		3774	978	245	85	57	81	17	2.43				52.89					
73 B	passing	0.99	1.51	1.98	2.5	3.1	3.74	5.0	6.3				13.5			1.36	2.7	6.3
		3908	1211	454	216	159	128	28	2.43				61.06					
74 A	original	0.95	1.45	1.9	2.4	3.0	3.6	4.8	6.0	7.5	9.0		9.42					
		2911	795	264	102	124	89	50	9.6	7.1	1.21		43.74			1.73	3.7	12.0
74 B	original	0.95	1.45	1.9	2.4	3.0	3.6	4.8	6.0	7.5	9.0	12.0	11.8					
		3276	995	490	177	208	149	80	12	12.1	6.1	1.21	53.86					
74 A	passing	0.95	1.45	1.9	2.4	3.0	3.6	4.8					6.54					
		2087	544	211	85	29	33	1.21					29.70			1.21	2.2	4.8
74 B	passing	0.95	1.45	1.9	2.4	3.0	3.6	4.8					9.99					
		3525	762	242	85	68	59	7					45.48					
75 A	original	0.95	1.45	1.9	2.4	3.0	3.6	4.8	6.0	7.5	9.0	12.0	9.7					
		2729	998	323	159	121	107	36	31	4.85	4.85	1.21	44.17					
75 B	original	0.95	1.45	1.9	2.4	3.0	3.6	4.8	6.0	7.5	9.0		8.35			1.79	4.2	12.0
		2049	906	330	153	175	115	45	16	4.85	5.89		39.00					
75 A	passing	0.95	1.45	1.9	2.4	3.0	3.6	4.8	6.0	7.5			9.5					
		2800	750	291	148	74	66	24	2.43	1.21			43.17					
75 B	passing	0.95	1.45	1.9	2.4	3.0	3.6	4.8					7.54			1.37	2.3	7.0
		2003	801	352	136	63	63	13.6					34.32					
76 A	original	0.95	1.45	1.9	2.4	3.0	3.6	4.8	6.0	7.5	9.0		12.4					
		3735	1053	386	141	109	109	59	30	4.86	5		56.22					
76 B	original	0.95	1.45	1.9	2.4	3.0	3.6	4.8	6.0	7.5	9.0		12.4			1.67	3.5	9.0
		3371	1160	425	233	211	149	41	12	8.52	7		56.18					
76 A	passing	0.95	1.45	1.9	2.4	3.0	3.6	4.8	6.0	7.5	9.0		10.9					
		3036	1074	391	124	158	126	53	14.6	6.2	2.43		49.85					
76 B	passing	0.95	1.45	1.9	2.4	3.0	3.6	4.8	6.0	7.5	9.0	12.0	15.3					
		4779	1235	437	163	163	124	37	17.2	9.9	2.0	1.21	60.68					

Table 1 is a complete count for experiment 68A (original cloud) showing, in brackets, the numbers interpolated by the method of the weighted mean. The focal length calculation for obtaining the original drop diameter, the method of summing and the working out of the distributions are also seen.

Table 2 summarizes the results of similar calculations for all the successful experiments. From these results smooth number distribution curves were plotted on logarithmic probability paper, and from these the percentage by number in each of the following size ranges was read:

0.4 to 2.0, 2.0 to 4.0, 4.0 to maximum (μ).

Multiplying by the mean number of drops per field and comparing the corresponding ranges for original and passing clouds led to the percentage penetration figures given in table 3. The percentage penetration by mass for the whole cloud is also given.

The 50 % diameters are those above and below which equal total masses or numbers of drops exist. The mean mass diameter is that which would be necessary if a given mass of liquid were dispersed to a number of equal-sized drops instead of to an equal number having the observed size distribution.

6. EXPERIMENTAL RESULTS

Experiment 76 was a control in which the original cloud was drawn at 2.0 l./min. through a circuit identical in every way with that used at other times except that there was no rabbit. The results show that the original cloud passed through the cannula and sampling tube with no appreciable loss, but their resistance was not negligible and was measured separately at different rates of flow. All figures given in this paper refer to the nose alone, this correction having been applied.

The results for experiment 71 show an impossibly high penetration in the largest size range. This experiment was done at a low flow and a low resistance so the penetration was large. The error probably arose from the sizing of the largest drops which penetrated to an unusually great extent; as a result the value 91 % for the mass penetration may be high by an amount not exceeding 10 %.

Resistance measurements were made in all cases at the time of sampling and, in the case of experiments 73, 74 and 75, immediately afterwards over a range of flows. The curves are plotted on figure 5. Readings were taken up and down the flow range and were reproducible.

Owing to the variation of individual animals, a separate animal being necessary for each experiment, a better correlation between penetration and resistance is found than between penetration and flow. From figure 5 it will be seen that resistance correlates with both the maximum drop penetrating and with the percentage mass penetration. It is also evident that, in general, the higher the flow the better is the filtration; this fact is in accord with the theory that deposition in the nose is brought about by the inertia of the droplets sufficing to carry them across the streamlines of flow into contact with the bounding walls of the air channels.

Details of the percentage penetration of various size ranges and other data are given in table 3. Summarizing broadly it can be stated that at ordinary rates of flow of 1–2 l./min. (corresponding to respiration rates of 0.5–1 l./min.), the nasal resistance is likely to be between 0.5 and 4.0 cm. water for most rabbits. All droplets above 7μ diameter will be removed by the nose on inhalation. About half the 3μ diameter drops will penetrate to the lungs, and practically all the 1.5μ ones and smaller. These experiments were conducted with steady air flows, whereas, in nature, respiration through the nose is intermittent. Since the terminal velocities of drops of the sizes in question are negligibly small and the purely mechanical effect of drop deposition is under review, it is thought that the findings give a good indication of droplet or particle deposition in the natural state. It is probable that all droplets or particles which come into contact with the moist internal surfaces of the nasal cavity would be retained.

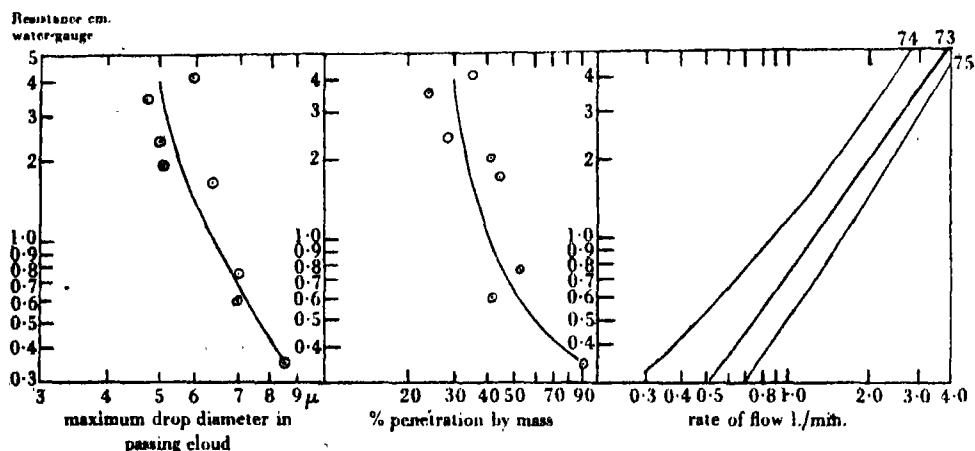


FIGURE 5. Experimental results for penetration of drops and resistance.

TABLE 3. SUMMARY OF EXPERIMENTAL CONCLUSIONS

experiment number	75	74	73	72	71	70	69	68	76 (control)
resistance cm., water gauge	0.6	3.5	1.7	4.2	0.35	0.75	2.4	2.0	0.5
flow, l./min.	1.0	2.0	2.0	1.5	0.3	0.3	1.0	1.0	2.0
maximum diameter penetrating	7.0	4.8	6.3	6.0	8.5	7.0	5.0	5.0	11.0
percentage penetrations by number									
range of diameters:									
0.4–2.0	100	82	90	92	90	77	56	31	106
2.0–4.0	57	37	64	76	88	73	37	46	100
4.0–max.	21	12	28	6.4	128	33	13	5.8	95

It was noted that the rhythmic twitching of the nostrils in sympathy with respiration was evident after the trachea had been cut and the animal was breathing through the cannula.

7. GENERAL DISCUSSION

(a) Anatomical structure

Figure 6 shows, in a diagrammatic manner, the main internal features of the nose. By using a planimeter on large photographic enlargements ($\times 12.5$) of transverse sections of the nose the cross-sectional areas of the free spaces in the channels have been measured. The area of each nostril is about 0.11 cm^2 . Each

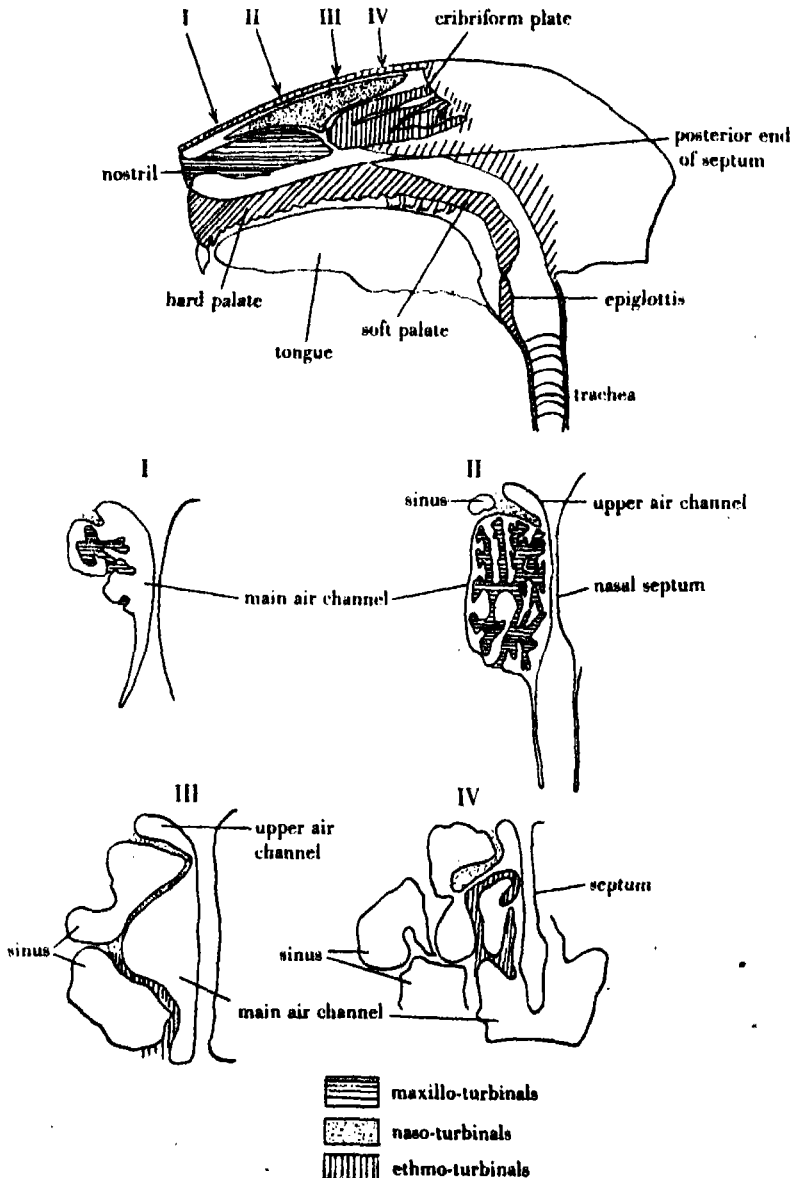


FIGURE 6. Structure of the nose of the rabbit (diagrammatic).

channel expands posteriorly, but the complex structure of the maxillo-turbinals also develops and occupies the central part of the cavity with its many ramifications. At section (i) the free-space area of each half of the nose is 0.12 cm.^2 . At section (ii) the area is 0.18 cm.^2 . Between (i) and (ii) it will be seen that the naso-turbinal extends inwards until it comes practically into contact with the nasal septum and isolates a small upper or dorsal channel. At section (ii) the area of this is 0.02 cm.^2 and that of the main channel 0.16 cm.^2 . Air passing through the main channel has to penetrate the interstices of the maxillo-turbinal. At section (iii) the upper and main channels are still distinct, and the latter, now freed of the maxillo-turbinal, has an area of 0.22 cm.^2 . The cross-section of the upper channel remains approximately constant. In section (iv) it will be seen that the main channel has moved ventrally and the two sides have run together, the septum being detached from the palate; the upper channel has debouched, ventrally, into the folds of the ethmo-turbinals. This region of the main channel is immediately anterior to the naso-pharynx and the total area (both sides) is 0.35 cm.^2 . The cross-section of the naso-pharynx is further reduced, prior to opening up into the trachea, to a minimum area of 0.19 cm.^2 .

(b) *Air flow*

The course of the main air flow is shown up well in figures 7*a* and 8*a* (plates 2 and 3), which are radiographs of a rabbit in which the cavities have been rendered radio-opaque by the injection of lipiodol. Figures 7*b* and 8*b* are control radiographs of the normal animal. The animal was kept lightly anaesthetized with nembutal and breathing through a cannula; lipiodol was injected upwards into the nose from the trachea. The rabbit was rotated to distribute the oil and injection was continued until it escaped from the nostrils. A small amount passed the epiglottis and outlined the space between the soft palate and the tongue. The septum and the maxillo-turbinals are clear in figure 7*a*, and in figure 8*a* some of the air spaces of the latter can be seen and some lipiodol has penetrated to the deep cavity above the ethmo-turbinals. The film of oil in the thin space between the septum and the ventral ethmo-turbinals is less clearly marked. None has penetrated to the small dorsal air channel.

Assuming an actual inflow of 1.5 l./min. the mean air speed at the various sections can be computed from the areas given below:

locality	total area A cm. ²	mean air speed cm./sec.
nostrils	0.22	114
section (i)	0.24	104
" (ii)	0.36	70
" (iii)	0.44	57
" (iv)	0.35	71
naso-pharynx	0.19	132

Considering the small single channel of the naso-pharynx which takes the total flow it is of interest to work out the Reynolds number, vd/ν , where v is the velocity, d the diameter and ν the kinematic viscosity of air. Thus, where Q is the total flow in c.c./sec.,

$$vd/\nu = Q \sqrt{\left(\frac{4A}{\pi}\right)} / A\nu = Q \sqrt{\left(\frac{4}{\pi A}\right)} / \nu = \frac{25}{0.15} \sqrt{\left(\frac{4}{0.19\pi}\right)} = 430.$$

This is the portion of the main channel, posterior to the maxillo-turbinals, where turbulent flow would be most likely to develop, but the value of the Reynolds number shows that the stream is laminar. Upon this basis it is possible to obtain a rough idea of the resistance to flow through the posterior portion of the nose by replacing it by a tube 5 cm. long and 0.25 cm. radius. The pressure drop across such a tube when the incoming air has negligible kinetic energy is given by the formula

$$P = \frac{8\eta Q}{\pi r^4} (l + \rho Q/8\pi\eta),$$

where η is the viscosity of air, ρ its density, l the length of the tube and r the radius. Putting in the values for air at 20° C and 1 atm. this reduces to

$$P = 0.000464 \frac{Q}{r^4} (l + 0.263Q).$$

The first term represents the pressure used in overcoming viscous traction and the second that expended in giving kinetic energy to the air.

For $l = 5$ cm., $r = 0.25$ cm., $Q = 1.5$ l./min., then

$$P = 14.9 + 19.6 = 34.5 \text{ dynes/cm.}^2 \text{ or } 0.035 \text{ cm. water.}$$

Now consider the flow through the maxillo-turbinals. As a rough approximation these can be replaced by a bundle of forty capillary tubes in parallel, each of radius 0.037 cm. and length 1.8 cm. Then the total free-space area is 0.17 cm.² per nostril, of the same order as the measurements. The above formula can now be applied with $Q = 12.5/40$ c.c./sec., giving

$$P = 140 + 5 = 145 \text{ dynes/cm.}^2 \text{ or } 0.15 \text{ cm. water.}$$

This resistance is about half the smallest measured value, but its magnitude depends considerably on the clearance between the bony structures or upon the analogue, the tube radius, r . It is at least clear that the bulk of the resistance to flow is located across the maxillo-turbinals, and that most of the stream must proceed through them.

Air which enters the upper channel during inhalation passes along the dorsal surface of the naso-turbinal and flows back into the main stream at right angles by passing through the slit-like space, about 1 cm. long (anterior-posterior), between the inner surfaces of the lower ethmo-turbinals and the nasal septum, which is

roughly central on section (iv). This slit is quite thin, probably about 0.2 mm. in some places, but owing to its length and the small cross-section of the upper channel which feeds a fraction of the inspired air, the air speed across the ethmo-turbinals must be quite low. Owing to the low air speeds involved the partition of air between the upper and main channels would be governed by their relative resistances and not, it is thought, by the directive action of jets. Hence air probably reciprocates in the upper channel during respiration. This is apparently employed to excite the olfactory sense organ located posteriorly on the ethmo-turbinals. It is difficult to see how the twitching of the nostrils during normal respiration could affect the flow through the upper channel, since its anterior junction with the main channel is surrounded by bony structures.

(c) Filtration

Interest in this connexion again centres upon the maxillo-turbinals. This structure may serve as a unit for warming and humidifying the inspired air as well as a dust filter. The clearance between the lamellae of the structure varies from about 0.5 mm. downwards with some channels as narrow as 0.1 mm. It is upon the walls of these narrow passages that the larger drops deposit. A drop of radius a can be projected through still air for a distance x when it has an initial velocity v by virtue of its kinetic energy which enables work to be done against the viscous drag of the surrounding air.

For moderate velocities and in the range of diameters considered, x can be calculated from the following formula, deduced from the ordinary principles of dynamics and Stokes's law of fluid resistance,

$$x = 2a^3 v \rho' / 9\eta,$$

where ρ' is the density of the drop or particle. For $v = 70$ cm./sec. the following figures have been calculated and are relevant to flow in the channels of the maxillo-turbinals:

distance x (mm.)	0.07	0.12	0.19	0.27
drop diameter $2a$ (μ)	6	8	10	12

It is thus evident that drops above about 7μ would possess enough kinetic energy to traverse distances comparable with the channel width so that deposition would be possible where the walls projected or curved in any way. A percentage of smaller drops which were closer to the walls would similarly be removed, but the rest, once clear of the turbinals, would pass into the lungs. Particles below 1.5μ diameter will penetrate freely unless they are so small that diffusion to the walls is appreciable during the short time of transit through the nasal channels. A good estimate of the maximum size at which this mechanism is effective can be obtained as follows.

If the diffusing particles are present in uniform size and distribution in a cloud of gas passing through a tube, the walls being supposed to have perfectly absorbing

surfaces, it is possible to calculate the ratio of the mean outlet concentration, \bar{c} , to the initial concentration c_0 . For laminar flow through the tube and neglecting end effects on the velocity profile,

$$\bar{c}/c_0 = 0.819 \exp(-14.6272\Delta) + 0.0976 \exp(-89.22\Delta) + 0.01896 \exp(-212\Delta) + \dots,$$

where $\Delta = \pi D l / 4Q$, D being the coefficient of diffusion for the particles, l the tube length and Q the mean rate of flow. This equation has been deduced from the result for an analogous problem in heat transfer which is dealt with in the work *Modern developments in fluid dynamics* (edited by S. Goldstein, 2, 619, 1938). It will be observed that the expression is independent of the diameter, the slower velocity in a wide tube exactly compensating for the greater distance through which particles must diffuse to reach the wall; at the same time, since \bar{c}/c_0 diminishes rapidly with increasing values of Δ , it is clear that absorption in the multiple channels of the maxillo-turbinals will greatly preponderate over that occurring elsewhere.

For 50 % absorption $\bar{c}/c_0 = 0.5$, and Δ , from the above equation, is 0.034. Substituting the usual values it follows that

$$D = 4Q\Delta/\pi l = \frac{4 \times 12.5 \times 0.034}{40 \times 1.8 \times \pi} = 0.0075 \text{ cm.}^2/\text{sec.}$$

Einstein's equation, corrected by the slip factor, F , enables the diameter of particles having this value of the diffusion coefficient to be obtained. From a review of the best data for the slip factor, then (Davies 1945)

$$F = 1 + 1/pa(6.32 \times 10^{-4} + 2.01 \times 10^{-4} \exp(-2190\pi p)),$$

where p is the air pressure in cm. of mercury and a the particle radius. By Einstein's theory

$$\frac{2a}{F} = \frac{kT}{3\pi\eta D} = \frac{1.381 \times 10^{-16} \times 293}{3\pi \times 0.00018 \times 0.0075} = 0.32 \times 10^{-8} \text{ cm.},$$

where k is the Boltzmann constant and T the absolute temperature. Solving the Einstein and the slip equations simultaneously by a graphical method, then

$$2a = 0.0026\mu \text{ or } 26 \text{ \AA.}$$

The weight of such a small particle is $9200\rho' \times 10^{-24}$ g. If it was a single molecule of unit density the molecular weight would be 5500.

The corresponding figures for 10 % penetration are $\bar{c}/c_0 = 0.1$, $\Delta = 0.143$, $D = 0.0316$, $2a/F = 0.0754 \times 10^{-8}$, $2a = 0.0012\mu$ or 12 \AA. Weight $\approx 905\rho' \times 10^{-24}$ g.; molecular weight about 550.

Molecular weights of the same order are also given by the approximate formula $D = 0.74/\sqrt{M}$.

These calculations are of some importance, since they show that gases of low molecular weight will be absorbed in the nose unless the rate of diffusion to the surfaces of the cavity is diminished. Any one of a number of factors which govern

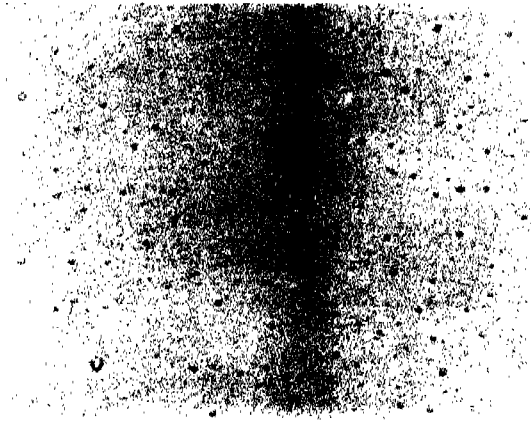


FIGURE 4b

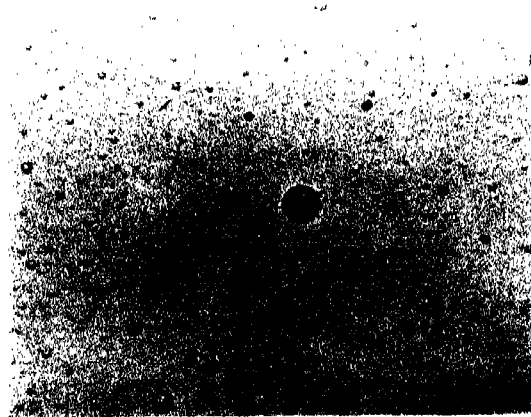


FIGURE 4a



FIGURE 7a



FIGURE 7b



FIGURE 8b



FIGURE 8a

the solution, reaction or disposal of absorbed gas might bring this about if an accumulation developed at some stage. The effect of this would be passed back to the surface where increased surface concentration of dissolved or adsorbed molecules would result in a higher concentration in the adjoining vapour layer and hence in slower diffusion to the surface on account of the diminished concentration gradient in the vapour phase. The control of absorption may rest with diffusion for some gases. These will be absorbed in the nose, but in other cases physical chemical factors within the tissues will be paramount; such gases may penetrate the nose and reach the lungs.

Using similar arguments to the above it can be concluded that given moist surfaces to the walls of the cavities the inflowing air must become saturated with moisture.

Finally, it should be remembered that the structure of the maxillary turbinates in the rabbit is exceptional; they are much simpler in man or in the goat, for example, so that in these species a greater chance of fine particles or molecules reaching the lungs would be anticipated. This might not be the case for large particles on account of the higher air flows but the channel sizes would have to be considered in relation to these.

This work was planned and carried out at a Ministry of Supply Research Establishment and arose out of another problem which was under investigation there. Thanks are due to the authorities for permitting publication.

The author is indebted to Dr E. Boyland, who suggested the investigation, for preparing and handling the rabbits for the penetration experiments and for general discussion of the problem; to Major H. M. B. Adam for preparing the animals for radiographic examination and for performing an instructive dissection; to Major R. H. D. Short for the loan of a very fine set of nasal sections; to Mr W. L. Dennis for developing the liquid flowmeter and for technical assistance; to Mr A. H. Rowe who constructed the sedimentation cells; and to Miss D. E. Thorne who carried out the computations.

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DESCRIPTION OF PLATES 1-3

FIGURE 4. $\times 380$. Deficiency of small drops noted in the region about large drops. 1.1μ drop = 1 mm. Experiment 28.

FIGURE 7. (a) Radiograph of the nasal cavity of the rabbit outlined with lipiodol. (b) Control.

FIGURE 8. (a) Radiograph of the nasal cavity of the rabbit outlined with lipiodol. (b) Control.

The differential effect of synthetic plant growth substances and other compounds upon plant species

I. Seed germination and early growth responses to α -naphthylacetic acid and compounds of the general formula $\text{arylOCH}_2\text{COOR}$

BY W. G. TEMPLEMAN AND W. A. SEXTON

(Communicated by Sir Henry Dale, P.R.S.—Received 8 November 1944)

[Plate 4]

Laboratory and small-scale experiments in soil are described in which α -naphthylacetic acid in solution, incorporated dry in fine sand and in ammonium sulphate, has been applied to oats (*Avena sativa* L.) and yellow charlock (*Brassica Sinapis Visiani*) at seed sowing and during early growth stages. Concentrations which kill the charlock leave the oats unharmed. Details are given of other weeds which react similarly to yellow charlock, whilst other cereals (wheat, barley and rye) behave like oats.

The chemical syntheses of a number of compounds of the general formula $\text{arylOCH}_2\text{COOR}$, where the aryl radicle was benzene or naphthalene, with and without substituents, and *R* was hydrogen, an alkali metal or an alkyl radicle, are described.

The biological examination of these compounds is described and certain characteristics of the more active ones indicated and discussed.

The practical importance of this discovery in the field of weed-killers which selectively eradicate weeds from cereal crops is pointed out. The incorporation of active materials in fertilizers for cereal crops is suggested. A programme of research on this subject is proceeding.

The study of the effects of plant growth substances at Jealott's Hill Research Station was originally undertaken to determine whether the organic matter applied to or present in soils owed any of its beneficial properties to the presence of plant growth substances such, for example, as β -indolylacetic acid. Plants have been treated with crude extracts of farmyard manure, composts and fertile soil and with pure growth substances. The experiments with pure growth substances (Templeman 1939; Templeman & Marmoy 1940) demonstrated no increase in the growth of plants as measured by the dry-matter production but, in common with a number of other investigators, it was observed that the highest concentrations used (especially of α -naphthylacetic acid) depressed the growth rate. Dr R. E. Slade suggested that these compounds might give different depressing effects on the growth rates of different plants, and that new methods of selectively influencing plant associations might result from the use of these compounds or others known to affect cell functions. This suggestion received support from the fact that the root initiating response of cuttings to α -naphthylacetic acid varied greatly from species to species (e.g. Metcalfe & Templeman 1939), and from the experiments on total growth carried out between 1937 and 1940. The first experiments of the investigation begun in 1940 with α -naphthylacetic acid showed that such variations with species did exist, and the programme was then extended by the synthesis of more active and more readily prepared compounds. Description is given of these experiments and of the subsequent biological work.

Since writing this paper we have seen a publication by Hamner & Tukey (1944) on *The Herbicidal Action of 2:4 dichlorophenoxyacetic and 2:4:5 trichlorophenoxyacetic acid on Bindweed*. The former of these two substances will be found in this paper classed among the compounds more active than α -naphthylacetic acid.

The biological work was carried out at Jealott's Hill Research Station and the chemical work in the Research Laboratories of the Dyestuffs Division of I.C.I. Ltd. at Manchester.

PART 1. THE DIFFERENTIAL EFFECT OF α -NAPHTHYLACETIC ACID ON THE GERMINATION OF OAT (*AVENA SATIVA* L.) AND YELLOW CHARLOCK (*BRASSICA SINAPIS VISIANI*) SEED

In the first place it was necessary to determine whether synthetic growth substances had a differential effect upon the germination and growth of different plant species. The first experiments carried out during 1940 used α -naphthylacetic acid or its sodium salt, which were known to bring about epinastic responses and to stimulate the initiation of roots on stems. The first two species—the oat (*Avena sativa* L.) and yellow charlock (*Brassica Sinapis Visiani*)—were chosen because of the practical value of such an investigation, since yellow charlock is the commonest annual weed of spring-sown cereals in this country.

Experiments were carried out upon seed sown in boxes of soil out of doors and upon seed placed upon moist filter paper in the laboratory.

Experiment 1 (a) Soil experiments

Wooden boxes 12 × 12 × 4 in. were filled with seeding compost made according to the John Innes Horticultural Institution formula and sown with oats (in two rows 6 in. apart) and yellow charlock (broadcast) on 23 August 1940. The boxes were watered and the following treatments applied the same day:

(1) Control—sand only			
(2) α -naphthylacetic acid	10 mg. per box	applied dry in 6 g. dry sand	
(3) " "	20	" "	" "
(4) " "	50	" "	" "
(5) " "	100	" "	" "
(6) " "	250	" "	" "
(7) Control—water only			
(8) α -naphthylacetic acid	10 mg. per box	applied in solution of 250 c.c. water	
(9) " "	20	" "	" "
(10) " "	50	" "	" "
(11) " "	100	" "	" "
(12) " "	250	" "	" "
(13) Control—water only			
(14) α -naphthylacetic acid	10 mg. per box	applied as spray in 10 c.c. water	
(15) " "	20	" "	" "
(16) " "	50	" "	" "
(17) " "	100	" "	" "
(18) " "	250	" "	" "

There were four replicate boxes for each treatment.

Germination counts were made at daily intervals for both oats and charlock. The results for 20 September 1940 are representative and are given in table 1.

TABLE 1. RESULTS OF EXPERIMENT 1

treatment	mean no. charlock plants per box, 20. ix. 40	percentage reduction of charlock germination	mean no. oat plants per box, 20. ix. 40	average height of oat plants, 20. ix. 40 in.
1	14.3	—	38.5	5.9
2	15.5	0	38.8	4.9
3	13.3	7	38.3	6.0
4	5.8	59	38.0	5.8
5	6.3	56	39.8	5.8
6	2.0	86	39.3	4.4
7	13.0	—	40.3	5.9
8	10.3	21	39.8	5.5
9	8.5	35	39.3	5.1
10	2.8	79	39.3	5.4
11	3.5	73	40.0	4.5
12	2.3	82	38.8	4.0
13	15.8	—	39.5	5.9
14	17.5	0	39.3	5.5
15	9.0	43	39.8	5.8
16	11.5	27	40.0	5.5
17	3.3	79	39.0	5.1
18	2.5	84	39.5	4.8

It is quite clear from this experiment that applications of 50–250 mg. per box of α -naphthylacetic acid (especially where applied by watering-on a solution) have markedly reduced the germination of the charlock without reducing that of the oats. At highest rates of application the rate of growth of the oats was slightly retarded as shown by the height measurements, but this difference from the controls gradually disappeared.

Experiment 2

A second box experiment was laid down to investigate further this differential effect on the two species used in the previous experiment. All the boxes were sown with oats as before on 16 September 1940. Half were sown with yellow charlock on the same day and the other half on 24 September 1940 when the oats were just showing through the soil. Treatments were applied on 26 September 1940 when the oats were $\frac{1}{2}$ in. high and the charlock germinating on the first series, and this time the α -naphthylacetic acid was applied at 50, 100 and 250 mg. per box in dry sand, as a sprayed solution and mixed with ammonium sulphate as a carrier. This latter method was adopted, since for practical agricultural purposes it would be suitable since it is normally applied in spring as a top-dressing to cereal crops.

The full list of treatments was as follows:

A.	(1) Control—sand only			
	(2) α -naphthylacetic acid	50 mg. per box	}	Dry in 5 g. sand per box
	(3) "	100 "		
	(4) "	250 "		
	(5) Control—water only			
	(6) α -naphthylacetic acid	50 mg. per box	}	Sprayed in solution 10 c.c. per box
	(7) "	100 "		
	(8) "	250 "		
	(9) Control—ammonium sulphate only			
	(10) α -naphthylacetic acid	50 mg. per box	}	Dry in 1.17 g. ammonium sulphate
	(11) "	100 "		
	(12) "	250 "		
B.	(13) Control—sand only			
	(14) α -naphthylacetic acid	50 mg. per box	}	Dry in 5 g. sand per box
	(15) "	100 "		
	(16) "	250 "		
	(17) Control—water only			
	(18) α -naphthylacetic acid	50 mg. per box	}	Sprayed in solution 10 c.c. per box
	(19) "	100 "		
	(20) "	250 "		
	(21) Control—ammonium sulphate only			
	(22) α -naphthylacetic acid	50 mg. per box	}	Dry in 1.17 g. ammonium sulphate per box
	(23) "	100 "		
	(24) "	250 "		

A. Treatments 1–12: oats and charlock sown together.

B. Treatments 13–24: oats sown first, charlock 8 days later.

Again there were four replicates for each treatment; daily germination counts and periodic height measurements were made.

The results for oat germination counts of 2 October 1940, oat height measurements for 1 November 1940, and charlock germination counts for 22 October 1940 are given in table 2.

The charlock which did germinate on the treated boxes in this experiment grew very slowly compared with that of the control boxes. As plate 4 shows, the differences between the controls and treated boxes were more marked than the figures indicate. This experiment confirms the first one and clearly shows that α -naphthylacetic does have a differential effect upon the germination of yellow charlock and oats.

In connexion with this effect two other experiments were carried out in boxes of soil, but are not described in detail. The first of these showed that the common weed-killers, sodium chlorate, sodium arsenite, copper sulphate and dinitro-o-cresol, did not have this selective action on germination of these two species when applied at similar rates to the α -naphthylacetic acid. The second showed that the root crops, mangolds and sugar beet, reacted similarly to the yellow charlock and not like the oats.

TABLE 2. RESULTS OF EXPERIMENT 2

treatment	mean no. charlock plants per box, 22. x. 40	percentage reduction of charlock germination	mean no. oat plants per box, 2. x. 40	mean height of oat plants 1. xi. 40 in.
1	44.8	—	33.3	7.1
2	48.0	0	34.8	6.6
3	39.8	11	37.0	6.8
4	31.8	29	35.8	6.5
5	49.3	—	33.8	6.4
6	39.0	21	35.3	6.6
7	38.0	23	34.5	6.8
8	27.3	45	36.5	7.1
9	59.0	—	33.8	7.4
10	34.3	42	36.3	6.9
11	33.0	44	39.0	7.0
12	27.8	53	38.3	6.8
13	61.5	—	38.0	7.1
14	50.8	17	38.0	6.8
15	31.5	49	38.8	7.1
16	19.0	69	37.5	7.6
17	54.3	—	35.5	7.3
18	34.3	37	36.0	7.1
19	29.3	46	37.5	7.0
20	23.0	58	38.0	7.0
21	63.8	—	38.5	7.0
22	29.8	53	38.0	6.8
23	25.5	60	37.5	6.9
24	21.5	66	38.5	7.5

(b) Experiments on filter paper

In addition to the experiments in soil, laboratory experiments were also conducted in which seeds of the same species were germinated upon filter paper moistened with solutions of α -naphthylacetic acid. The actual equipment used was a Petri dish containing the solution, a glass plate ($\frac{1}{4}$ plate size) lying on the Petri dish, thick filter paper cut to the size of the plate with two tabs bent down into the solution to act as wicks, the seeds (50 for oats and 100 for yellow charlock) placed on the filter paper and covered by a small Copenhagen bell-jar. There were always four replicate units for each treatment. All yellow charlock seed used in the laboratory experiment was pre-treated in concentrated sulphuric acid for 5 min., thoroughly washed in calcium carbonate suspension, then in water and finally dried so as to increase the percentage germination of this seed.

Experiment 3

The experiment was set up on 9 January 1941, and daily germination counts were made until no increase occurred. This was until 27 January 1941, and the figures for this date are given in table 3.

TABLE 3. RESULTS OF EXPERIMENT 3

treatment		mean germination 27. i. 41	percentage reduction in germination
oats			
(1)	Control	48.0	—
(2)	α -naphthylacetic acid: 50 p.p.m.	48.0	0
(3)	100 p.p.m.	47.5	2
(4)	200 p.p.m.	46.5	3
(5)	300 p.p.m.	46.8	2
(6)	400 p.p.m.	39.5	18
yellow charlock			
(7)	Control	15.8	—
(8)	α -naphthylacetic acid: 50 p.p.m.	2.8	82
(9)	100 p.p.m.	1.0	94
(10)	200 p.p.m.	1.0	94
(11)	300 p.p.m.	0.0	100
(12)	400 p.p.m.	0.0	100

The results on the germination of these two plants are very striking. After germination the root growth of charlock was much restricted at the lowest concentration and practically nil at 200 p.p.m., the highest concentration at which germination took place. At first there was slower root and shoot growth for oats at all concentrations, even though germination was practically unaffected, but later the plants, although showing some epinastic response, made good recovery.

A repetition of experiment 3, but using sodium α -naphthylacetate instead of the free acid gave results which were exactly as in the previous one.

From these preliminary experiments it is obvious that α -naphthylacetic acid (or its sodium salt) has a much greater effect upon the germination and early growth of yellow charlock than it has upon oats. Other tests not described in detail showed that greater plantain (*Plantago major* L.) and yarrow (*Achillea Millefolium* L.) behaved very similarly to yellow charlock, and that other cereals (rye, barley and wheat) reacted like oats. The first point was thus established by these preliminary experiments that there are concentrations of α -naphthylacetic acid which much more seriously damage germination and early growth of some species than they do others.

At this stage it was decided that a chemical survey should be undertaken of related substances with a view to the discovery of more active and more readily prepared compounds. This work is reported in part 2 of this paper.

PART 2. THE SEARCH FOR ALTERNATIVE SUBSTANCES TO α -NAPHTHYLACETIC ACID

The guiding motive was an economic one, and in seeking alternative substances the chemical aspect of the problem prompted a search first amongst naphthalene derivatives which did not require for their synthesis the direct attachment of a carbon atom to the naphthalene nucleus. It is this feature of α -naphthylacetic

acid which rendered it economically unattractive for the purpose in view, namely, the development of a selective weed-killer for agricultural use. The discovery that β -naphthoxyacetic acid (Bausor 1939*a, b*) produced responses in plants broadly similar to those produced by α -naphthylacetic acid provided, therefore, a desirable chemical type, and it was quickly found that, like α -naphthylacetic acid, this substance had a pronounced effect on the germination of charlock at concentrations which had no effect on oats. The substance was, however, less potent than α -naphthylacetic acid, and this prompted a systematic examination of compounds of the general formula:



where the aryl radicle was benzene or naphthalene, with and without substituents, and R was hydrogen, an alkali metal or an alkyl radicle.

Preparation of compounds

(*a*) *Acids*. The following acids were prepared by methods described in the literature, and were characterized by their melting-points. The general preparative method involved condensation of the parent phenol, with chloracetic acid in the presence of alkali, though the dinitro compound was obtained by nitration of the unsubstituted phenoxyacetic acid:

phenoxyacetic	<i>p</i> -chlorophenoxyacetic
<i>o</i> -methylphenoxyacetic	2:4-dichlorophenoxyacetic
<i>m</i> -methylphenoxyacetic	2:4:6-trichlorophenoxyacetic
<i>p</i> -methylphenoxyacetic	4-chloro-3-methylphenoxyacetic
2:4-dimethylphenoxyacetic	<i>p</i> -nitrophenoxyacetic
2:5-dimethylphenoxyacetic	2:4-dinitrophenoxyacetic
3:4-dimethylphenoxyacetic	α -naphthoxyacetic
<i>o</i> -chlorophenoxyacetic	β -naphthoxyacetic

The following acids have not hitherto been described. They were prepared by condensation of the phenol with chloracetic acid in the presence of alkali and purified by crystallization from an appropriate solvent. Their constitution follows from the method of preparation and from the fact that they were all soluble in dilute sodium carbonate. As a further check upon the constitution of 4-chloro-2-methylphenoxyacetic acid, the only one of the new compounds which gave outstanding biological effects, it was subjected to analysis for chlorine. Found: Cl = 17.6 %; $\text{C}_9\text{H}_9\text{O}_3\text{Cl}$ requires Cl = 17.7 %.

acid	crystallized from	melting-point (°C)
2:5-dichlorophenoxyacetic	water	149
2:4-dichloro- α -naphthoxyacetic	not crystallized	135
1-chloro-2-naphthoxyacetic	aqueous alcohol	160-1
2-chloro-1-naphthoxyacetic	water	134
4-chloro-2-methylphenoxyacetic	benzene	118
<i>ar</i> -tetrahydro- β -naphthoxyacetic	alcohol	156

(b) *Esters, etc.* In order to determine whether ready solubility in water was an essential property of a substance having a powerful effect in preventing the germination of seeds, a series of esters of α - and β -naphthoxyacetic acids was prepared, together with the amide and nitrile of the latter. Ethyl β -naphthoxyacetate was made by esterification of the acid with ethyl alcohol as described in the literature, and the amide was obtained by treatment of the ester with ammonia and was converted to the nitrile by means of phosphorus pentoxide. These three compounds were characterized by their melting-points. Other esters of β -naphthoxyacetic acid (not described in the literature) were made the same way by boiling the acid in excess of the appropriate alcohol in the presence of a small quantity of sulphuric acid. Each was purified by an appropriate procedure, and their characteristics are indicated in the table below.

ester	physical state	physical characteristic
methyl	crystals (from methyl alcohol)	m.p. 75-7°
propyl (<i>n</i>)	liquid	b.p. 198°/10 mm.
propyl (<i>iso</i>)	low melting crystals	b.p. 192°/11 mm.
butyl (<i>n</i>)	liquid	b.p. 212°/15 mm.
butyl (<i>iso</i>)	liquid	b.p. 202°/12 mm.
cyclohexyl	crystalline solid	m.p. 52° b.p. 220°/12 mm.

The isopropyl ester, being of particular interest because of its high biological activity, was subjected to analysis. Found: C, 73.7; H, 6.8 %; $C_{15}H_{16}O_3$ requires C, 73.9; H, 6.6 %.

Ethyl α -naphthoxyacetate is described in the literature (Spica 1886) as a crystalline solid, m.p. 173-4°. This description is incorrect, and we record a figure of 55-6° for its melting-point. The compound was prepared in the same way as the above esters of β -naphthoxyacetic acid and purified by crystallization first from alcohol and then from light petroleum. Analysis gave: C, 72.2; H, 5.9 %; $C_{14}H_{14}O_3$ requires C, 73.0; H, 6.1 %.

PART 3. EXAMINATION OF COMPOUNDS SYNTHESIZED AND OTHER BIOLOGICAL EXPERIMENTS

The second part of the biological work consisted of the examination of the materials described in part 2 and of the determination of the reaction of other species both crops and weeds.

To examine the compounds which had been synthesized a dual sorting test was used as follows:

(a) *Laboratory*: a test under laboratory conditions in quadruplicate with seed of spring oats and yellow charlock on absorbent paper imbibing an aqueous

solution of 200 p.p.m. of the substance under test for a period of about 10 days. Only water-soluble compounds were subjected to this test.

(b) *Soil*: a test in duplicate in 12 × 12 in. boxes of soil sown with spring oats and yellow charlock and treated immediately after seed sowing with the dry material at a rate equivalent to 25 lb. per acre ground in sand. The activity was assessed after a period of 3 weeks. This test was conducted in the open and the boxes were watered after application of the material under test and subsequently as necessary. All compounds were tested in this way.

In the laboratory test daily germination counts were made and observations recorded of the appearance of the seedlings of both species. For the soil series, periodic germination counts of both species and height measurements of the oats together with visual observations were made. Control (untreated) and α -naphthyl-acetic acid treatments were included in each test of a group of compounds.

The results of these experiments indicate that the majority of compounds used at the experimental concentrations had a conspicuous depressing effect on germination and seedling growth of the yellow charlock with little or no effect on the oats. The effect upon the charlock was either the prevention of emergence of the radicle, or if there was radicle emergence then the reduction and arrest of growth of this organ. In the soil series the difference between the controls and the most active treatments was most marked, the treated boxes showing no weed growth or germination whatsoever.

It has been possible to divide the compounds tested into the following broad classes:

Class A (activity greater than α -naphthylacetic acid):

- o*-methyl phenoxyacetic acid (Na salt)
- m*-methyl phenoxyacetic acid
- p*-methyl phenoxyacetic acid
- 2:4-dimethylphenoxyacetic acid
- 2:5-dimethylphenoxyacetic acid
- 3:4-dimethylphenoxyacetic acid
- p*-chlorophenoxyacetic acid (Na salt)
- 2:4-dichlorophenoxyacetic acid
- 2:5-dichlorophenoxyacetic acid
- 4-chloro-2-methyl phenoxyacetic acid
- 4-chloro-3-methyl phenoxyacetic acid
- β -naphthoxyacetic acid (Na salt)
- 2:4-dichloro- α -naphthoxyacetic acid (Na salt)
- methyl β -naphthoxyacetate
- propyl (*n*) β -naphthoxyacetate
- propyl (*iso*) β -naphthoxyacetate
- butyl (*iso*) β -naphthoxyacetate

Class B (activity approximately equal to α -naphthylacetic acid):

o-chlorophenoxyacetic acid (Na salt)
 2:4:6-trichlorophenoxyacetic acid (Na salt)
ar-tetrahydro- β -naphthoxyacetic acid
 β -naphthoxyacetamide
 β -naphthoxyacetonitrile
 ethyl β -naphthoxyacetate
 butyl (*n*) β -naphthoxyacetate
 cyclohexyl β -naphthoxyacetate

Class C (some activity):

phenoxyacetic acid
p-nitro-phenoxyacetic acid (Na salt)
 2:4-dinitro phenoxyacetic acid (Na salt)
 α -naphthoxyacetic acid (Na salt)
 1-chloro-2-naphthoxyacetic acid (Na salt)
 2-chloro-1-naphthoxyacetic acid (Na salt)
 ethyl α -naphthoxyacetate

Class D (inactive):

5-chloro-*o*-cresol
 β -naphthol

It was evident that the derivatives of phenoxyacetic acid and β -naphthoxyacetic acid which appear in class A were most promising materials. Finer distinctions between the compounds were not possible in this first series of tests, but later experiments (to be published subsequently) indicate that 4-chloro-2-methyl phenoxyacetic acid and 2:4-dichlorophenoxyacetic acid are probably the two most active compounds in the present list.

The time of application of active material was at seed sowing in all the experiments described above. It was obviously necessary to determine whether applications made later in the life history of the two experimental plants also showed the selective action. In this connexion α -naphthylacetic acid (Na salt), *p*-chlorophenoxyacetic acid (Na salt) and 4-chloro-2-methyl phenoxyacetic acid when applied at 25 lb. per acre to yellow charlock in the two-leaf stage (two real leaves expanded in addition to the cotyledons) caused epinasty, suppression of growth and subsequently withering and death. Ethyl β -naphthoxyacetate, 4-methyl phenoxyacetic acid and 4-chloro-2-methyl phenoxyacetic acid showed very similar effects at 15 lb. per acre when the charlock had three or four real leaves. The oats were either undamaged or showed some small check in growth from which they appeared completely to recover.

It is clear, therefore, that the selective action resulting in the death of yellow charlock plants is not confined only to at-seeding applications. It was also essential to know how other weed species and other crops reacted to this treatment. Only

preliminary work has been possible on this aspect of the problem, but α -naphthylacetic acid (Na salt) in a laboratory test at 400 p.p.m. completely inhibited the germination of plantain (*Plantago major* L.) and yarrow (*Achillea Millefolium* L.), whilst ethyl β -naphthoxyacetate and 4-methyl phenoxyacetic acid seriously affected (at 200 p.p.m.) the early growth of corn buttercup (*Ranunculus arvensis* L.), fat hen (*Chenopodium album* L.), corn marigold (*Chrysanthemum segetum* L.), corn spurrey (*Spergula arvensis* L.) and field poppy (*Papaver Rhoeas* L.).

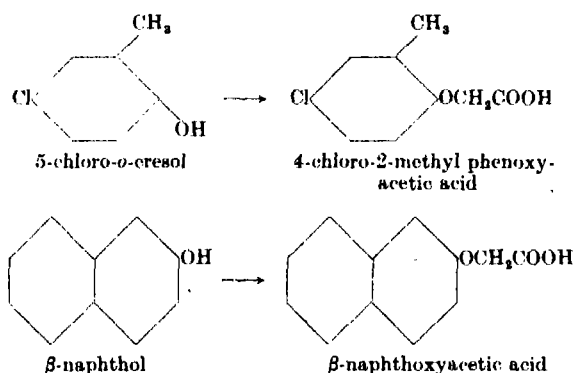
Wheat, barley and rye behaved very similarly to oats, but marrow-stem kale, mangold, sugar beet and flax were all seriously affected by applications of 25 lb. per acre of some of the substances in class A and class B. Further work is being done on the susceptibility of a wide range of crop plants to these substances, but it is evident that the cereal plants are more resistant to harmful effects than are a number of weed and other crop plants.

DISCUSSION OF RESULTS

The suggestions from earlier work that the concentration of α -naphthylacetic acid which prevents germination or seriously restricts seedling growth is different for different species has been investigated and proved to be true for the two species oats and yellow charlock. It is shown that the necessary concentration for yellow charlock is lower than it is for oats, and from the small amount of evidence available a similar state of affairs prevails for a number of dicotyledonous weeds and crops in contrast to other cereal species. Quite probably at higher concentrations still, the growth of the cereal would be restricted and the maximum differences between the responses of the two species will be where the growth reduction/concentration curves for them are most widely separated. The experiments described in this paper were evidently within this zone of wide separation. No effort had been made to search for small differences, and it is quite likely that such an investigation will reveal many different responses of both types and degree from different species. The effect is not confined to α -naphthylacetic acid. Some substances of the general formula $\text{arylOCH}_2\text{COOR}$ have proved more active than α -naphthylacetic acid in depressing yellow charlock growth whilst leaving the oats unharmed. In preliminary trials these two species also responded differentially to concentration of β -indolylacetic acid which occurs naturally.

The Boyce Thompson workers and others (Irvine 1938; Bausor 1939, 1939; Bausor, Reinhart & Tice 1940; Zimmerman & Hitchcock 1939; Zimmerman, Hitchcock & Wilcoxon 1939) have recorded some effects of β -naphthoxyacetic acid, and (Zimmerman & Hitchcock 1942) the substituted phenoxyacetic acids. β -naphthoxyacetic acid was shown to produce abnormal formative effects on intact growing plants and of the substituted phenoxyacetic acids which they used, 2:4-dichlorophenoxyacetic acid was proved to be extremely active in similar ways. The lower susceptibility of cereal seeds and seedlings to these substances when compared with other dicotyledonous plants has not, however, been previously reported.

For this particular effect which has been examined in our experiments, there are several interesting features which issue from the testing of a range of related chemical compounds. So far efforts to correlate chemical constitution and physiological properties of plant growth substances have failed. Considering the relationship between chemical constitution and physiological effect within the limited chemical sphere represented by compounds of the general formula $\text{arylOCH}_2\text{COOR}$ it is first of all important to note the inactivity of β -naphthol and 5-chloro-*o*-cresol, the parent phenols from which two of the most active compounds (β -naphthoxyacetic acid and 4-chloro-2-methyl phenoxyacetic acid) are derived:



The effects produced appear to be due to the molecule as a whole and several points may be noted:

(a) The greater activity of β -naphthoxyacetic acid over the α -isomer contrasts with the order prevailing in the known root stimulating properties of α - and β -naphthylacetic acids.

(b) Chlorination of the phenoxy compounds generally appears to increase their activity, whereas this is not so for chlorination of the naphthoxy compounds.

(c) The introduction of methyl groups into phenoxyacetic acid has a marked effect.

It is very interesting that the amide, nitrile and some of the esters of β -naphthoxyacetic acid are at least as active as the acid itself. These substances differ profoundly from the parent acid and its salts in their physical properties. They are relatively insoluble in water, have a higher volatility, and their solubility in lipoids is higher. The activity of a compound does not, therefore, appear to be entirely dependent upon its physical properties, though these may play an important contributory part. It is possible that the esters, etc., are simply hydrolysed to the free acid upon or before reaching the seat of the reaction, the active agent being the free acid.

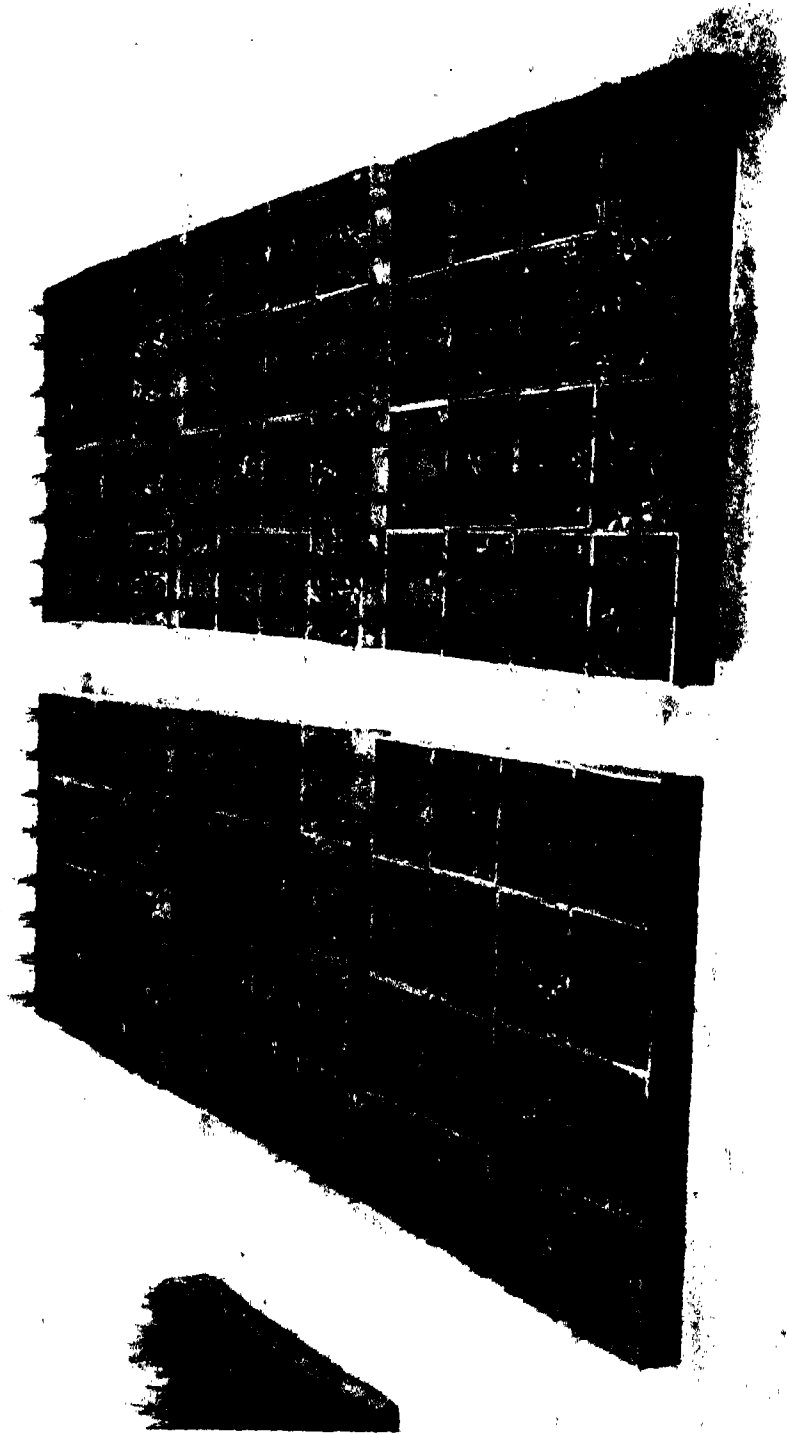
The results of the experiments so far show that the cereal plants are the least susceptible with non-cereals more easily damaged, and the work is being extended to discover whether the members of the Gramineae generally react like oats and whether variations in response are present in other non-cereal plants. A study of

the different characteristics of cereal and non-cereal seeds and seedlings is essential to any effort to discover the mechanism of the action of the aryloxyacetic acids. It was at first thought that these materials in some way interfered with the enzymic mobilization of the food reserves of the seed at and during germination, but the fact that intact and growing plants as well are stunted and the root growth drastically suppressed suggests that the effect is more general upon plant tissue. It is proposed to initiate studies of the effects of the most active substances upon the water relations, enzyme reactions and chemical composition of species of the two contrasting types. It seems that interference with some enzyme system or systems is a likely explanation, and therefore the effects of the aryloxyacetic acids will be tried upon some of the known enzyme reactions. Investigation of the anatomical, morphological and cytological effects is also being pursued.

The two species which have been used in these experiments differ widely in their responses to concentrations of the active substances. It is conceivable, however, that a range of differing degrees of response may be found when a large number of species has been examined, and it may be possible to classify them on this basis. It will be most interesting to discover what relations exist, if any, between a classification founded upon the reaction of species to growth substances such as the aryloxyacetic acids and the normal one based upon morphological characteristics.

This discovery of the differential depressing effects of these substances also raises the question whether the type and botanical composition of natural plant associations are due to or influenced by concentrations of substances with similar properties occurring under natural conditions. Such a possibility appears more real when it is realized that 0.1 p.p.m. in the growing medium of some of these active materials will practically suppress the growth of some species. Many other interesting results will be discussed in later papers of this series.

From the practical standpoint the differential response of plants to certain concentrations of the aryloxyacetic acids is most important. Two of the most common plant associations of agriculture consist of graminaceous plants growing together with non-graminaceous ones, to wit, the grass and legume species of pastures and the cereal and weed species of arable land. It is quite feasible that the legume content of a pasture sward may be reduced by light dressings of such materials as mentioned in this paper and the possibility of reduction, eradication or the prevention of infestation of cereal crops by dicotyledonous weeds is of supreme practical importance. A great deal of work upon the reaction of different weeds is necessary, but the value of a selective weed-killer working by adsorption through the roots as well as the leaves would be very great. The incorporation of such a material in the normal spring fertilizer dressing given to cereal crops, if effective in destroying annual weeds and preventing weed-seed germination during the early growth of the corn crop, would have immense beneficial possibilities. Field experiments are in progress and will be described in subsequent communications.



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EXPLANATION OF PLATE 4

Boxes from experiment 2 photographed 1 November 1940. Block on left. Charlock sown 8 days after oats. Treatments 1-12 front to rear. Block on right. Charlock and oats sown together. Treatments 13-24 front to rear. Four replicates side by side.

Control of nucleic acid charge on the X-chromosome of the hamster

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[Plate 5]

The differential segment of the X-chromosome of *Cricetus auratus* when its nucleic acid charge is reduced appears as a thin, unspiraled and understained thread. This happens under all conditions which lower the frequency of spermatogenesis and in proportion to their effect in doing so, viz. winter conditions (anoestrus), undernourishment, disease and old age. The frequency of the effect is variable, ranging from nil to 90 % of cells in a given testis. In up to 3 % of cells, the starvation of the differential segment is recognizable but incomplete. In 10 % the sex bivalent could not be recognized and its differential segment was assumed to be fully charged. When conditions lead to extreme nucleic acid depletion there is some failure of chiasma formation and metaphase pairing of both autosomes and sex chromosomes. This indirect deprivation of nucleic acid, like that directly induced by cold, suppresses spiralization and hinders division or separation of the chromosome threads. Extreme food starvation, combined with senility, stops spermatogenesis at the pachytene stage, presumably owing to protein as well as nucleic acid starvation.

The variability of nucleic acid charge in a warm-blooded animal as opposed to its regularity when reduced by cold in plants and Amphibia seems to be due to its dependence on the supply of ribose nucleic acid and not on the simpler chemical process of conversion of this supply into the deoxyribose form. The sensitivity of heterochromatin to variation in nucleic acid supply makes it possible to use its behaviour as an indicator of nucleic acid metabolism.

INTRODUCTION

The golden hamster (*Cricetus* or *Mesocricetus auratus*), a small rodent from Syria, shows remarkable behaviour at meiosis. In a proportion of spermatocytes the differential segment of one of the sex chromosomes, presumably the X, appears as a thin, unspiralized and understained thread at first and second metaphase (Koller 1938). Similar understained segments have been described in the chromosomes of *Paris*, *Fritillaria* and *Trillium* by Darlington & La Cour (1938, 1940, 1941) when the plants were kept at low temperatures. These authors showed that the segments which were negative to Feulgen's reaction were undercharged with desoxyribose nucleic acid in metaphase and overcharged in the resting stage: they were composed of heterochromatin. They suggested that the condition found in the hamster was similarly due to *nucleic acid starvation* of the heterochromatin.

I adopt the term 'starvation' used by the previous workers without any implication as to the means by which the depletion, deprivation, or mere deficiency of nucleic acid arises. In *Triton*, where low temperature is the determining condition, Callan (1942) has shown that it acts, not at mitosis, but only at meiosis, after a diffuse stage when a nucleic acid store in the heterochromatin is absent. In this case the starvation seems to be genuinely due to a lack of supply inside the nucleus, combined with lack of admission of its precursors from outside the nucleus. Again, Darlington & La Cour (1945) have shown that irradiation, which increases the ribose nucleic acid content of the cytoplasm, abolishes the nucleic acid starvation of the cold-treated heterochromatin in *Trillium*. The mechanism of nucleic acid deposition may, however, break down in many different ways. It seemed, therefore, worth while attempting to identify the factors which reveal this particular segment in the hamster and act as heterochromatin differentiators.

Observations which I have collected during the past six years indicate that several factors modify the exceptional nucleic acid cycle of the heterochromatin in the hamster. In young hamsters, kept on normal diet and at the peak of the breeding season, the sex bivalents and autosomes can rarely be distinguished in either shape or behaviour. But in old animals, or young ones out of season, the XY pair can be identified in the majority of spermatocytes by the thin, understained differential segment. It came to light incidentally, in 1938, that the hamsters which first showed the unspiralized segment of the sex chromosome in nearly all spermatocytes were not only old, but undernourished too. I therefore decided to investigate the role of four factors—age, breeding season, diet and disease—on the nucleic acid charge of the heterochromatin.

MATERIAL AND TECHNIQUE

The animals used for experiment were derived from a stock, the origin of which was described in the previous paper. They were all descendants of one female and her twelve young captured near Aleppo in 1930 and introduced into this country by Professor E. Hindle (Bruce & Hindle 1934).

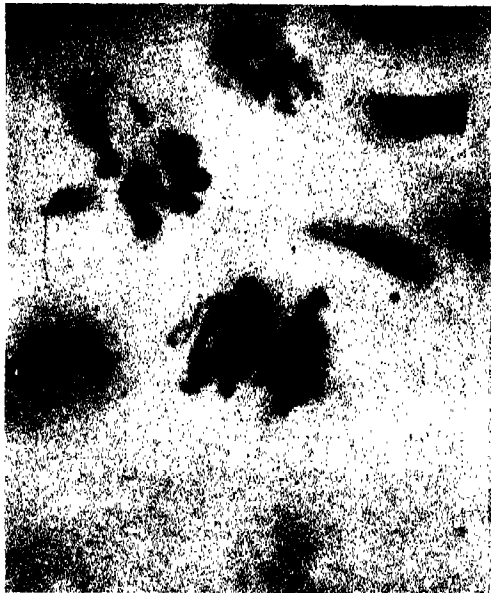


FIGURE 4



FIGURE 5



FIGURE 6

Over forty males were kept, two to four together, in wire cages measuring $35 \times 40 \times 20$ cm. The temperature of the well-lit breeding house varied usually between 20 and 23° C. It rose occasionally to 28° C in the summer and dropped to 18° C in the winter.

The two testes of the same individual (referred to as *a* and *b* in the tables) were fixed at different times. The animals recovered within 3-4 days from the effect of unilateral castration, and their mating habits remained as before. Pieces of testis were fixed for 6 hr. in Flemming's solution modified by Minouchi. Sections were cut at 20-25 μ , stained with Feulgen's basic fuchsin or gentian violet. Smear preparations were also made and stained with acetic lacmoid and orcein (Darlington & La Cour 1942). All these methods gave similar results.

Meiosis takes place in different parts of the testis at different times. The number of spermatocytes with and without the thin, unspiralized differential segment of the sex bivalent was counted in the seminiferous tubules. In about 10 % of cells the sex bivalent could not be seen, and these cells were counted as non-starved. There is variation in the frequencies between different regions in the same testis and between the two testes (table 1). This variation can be partly attributed to the fact that in about 3-5 % of cells the nucleic acid starvation of the differential

TABLE 1. THE FREQUENCIES OF SPERMATOCYTES SHOWING THE DIFFERENTIAL SEGMENT IN DIFFERENT REGIONS WITHIN THE SAME, AND BETWEEN THE TWO TESTES. ALL FIXATIONS DURING THE MATING SEASON

animal number	date of fixation	age of animal months	region	total number of cells	starved	
					number	%
right testis						
32	10. v. 42	6	A	72	12	16.6
			B	24	4	16.7
			C	35	8	22.8
			total	131	24	18.3
34	3. viii. 42	14	A	58	12	20.5
			B	75	21	28.0
			C	27	9	33.3
			total	160	42	26.2
left testis						
32	10. v. 42	6	D	18	2	11.6
			E	50	7	14.0
			F	47	10	21.2
			total	115	19	16.5
34	3. viii. 42	14	D	49	13	26.5
			E	65	20	30.7
			F	32	10	31.2
			total	146	43	29.4

segment is incomplete and these may have been classified variously. Allowing for this, variation of the frequencies is always within the range of sampling error in any individual testis; therefore only one region with cells in meiosis was analysed from each.

The behaviour of the differential segment

In the golden hamster with 38 chromosomes the differential segment cannot be identified at mitosis or during oogenesis (Husted, Hopkins & Moore 1945). In the striped hamster (*Cricetulus griseus*) with 14 chromosomes, a related species from China, the X and Y differ in size and are larger than any of the 16 autosomes (Pontecorvo 1943). At meiotic metaphase the differential segment varies greatly in both species. It is represented in some spermatocytes as a long, thin, slightly stained thread; the length of this thread is sometimes twice that of the pairing segments of the X and Y chromosome; in other cells it is short and thick (figure 1). Length, diameter and degree of staining vary together, the shorter the thread the greater its diameter and the deeper it stains. The shortening and thickening of a chromosome is known to be determined by spiralization which is conditioned in turn by the nucleic acid charge (Darlington 1942).

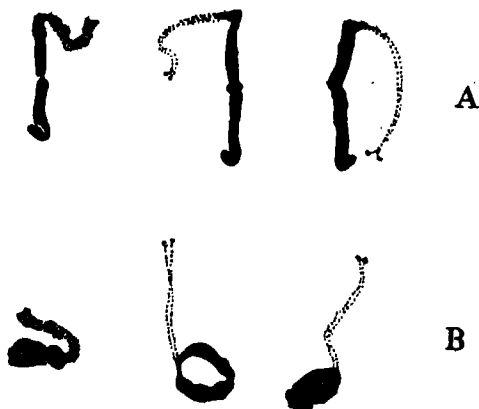


FIGURE 1. The asymmetrical (A) and symmetrical (B) sex bivalents showing the differential segment. $\times 2300$.

The differential segment may show extreme nucleic acid starvation in one cell, while its charge is normal in an adjacent cell. Between these two extremes every gradation can be seen in different spermatocytes, but the variation is of the same order in the two testes. Spermatocytes showing the starved differential segment may be grouped together or distributed at random within the seminiferous tubule. The degree of nucleic acid starvation within a cell must depend on developmental variations, but this is not reflected in any regular position of the starved cells within the seminiferous tubules. Spiralization fails only where nucleic acid starvation is extreme, as in polyploid species of *Trillium* and *Paris* where the amount of heterochromatin is small, or owing to the other special conditions already mentioned.

as in *Triton* (Callan 1942). In these organisms no intermediate nor variable degrees of spiralization have been found, but it should be noted that in all of them the determining factor is temperature, which, of course, cannot be used as an experimental agent in a mammal like the hamster. The variation in the hamster therefore seems to be due to a more complex and indirect control.

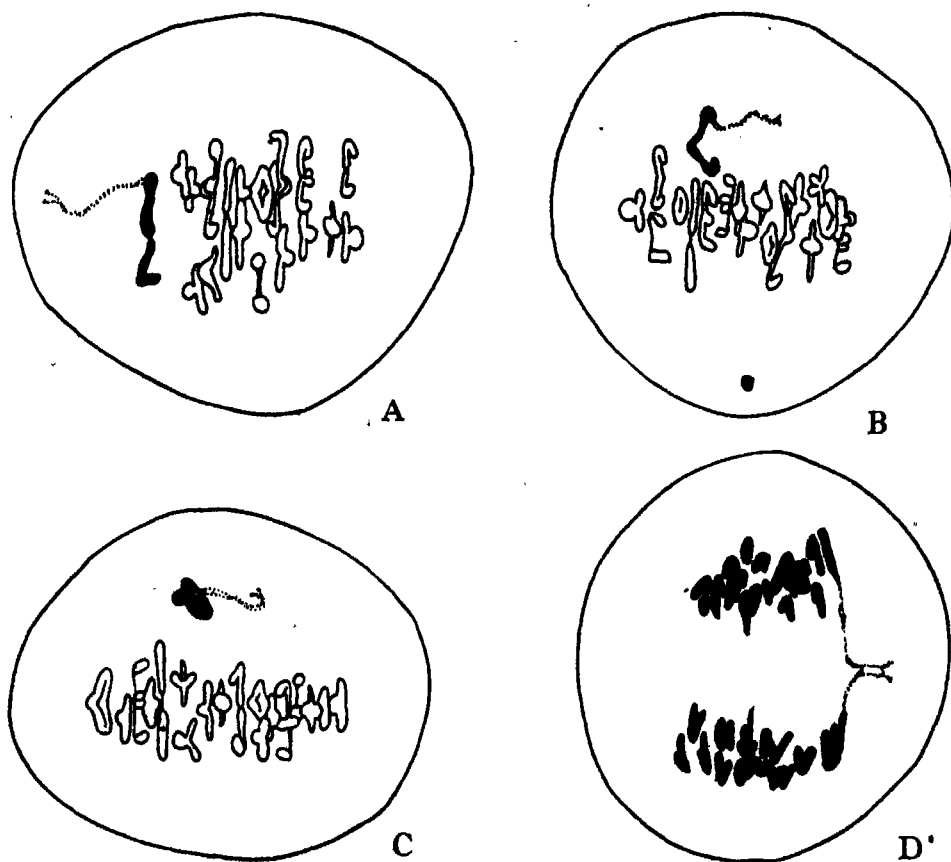


FIGURE 2. Sex bivalents with the unspiralized differential segment showing lack of co-orientation and congression at metaphase (A, B and C) and stickiness at anaphase (D). $\times 2300$.

Both types of sex bivalent, symmetrical and asymmetrical, with chiasmata on differential and non-differential sides of the centromere respectively, are formed during meiosis (figure 1 A and B; also see figure 12, diagrams, Koller 1938). When the differential segment is undercharged both types lie off the equatorial plate. The symmetrical XY bivalent is displaced towards the pole, the asymmetrical to one side of the plate (figure 2 A, B, C and plate 5, figure 4). The abnormal position and lack of co-orientation suggest a weakness of the centromere. Similar behaviour was observed in the heterochromatic B-chromosomes of maize (Darlington & Upcott 1941), and *Sorghum* (Darlington & Thomas 1941), and several other plants.

At first meiotic anaphase the ends of the thin and undercharged chromatids of the differential segment are sticky and remain associated as in cold-treated *Paris* (figure 2D).

Occasionally the sex chromosomes fail to pair. The differential segment was then always completely starved (figure 3A). In such cells and in no others autosomes were sometimes unpaired too and the spindle somewhat disorganized (figure 3B). Thus depletion of the heterochromatin is a symptom of a general disturbance of cell activity.

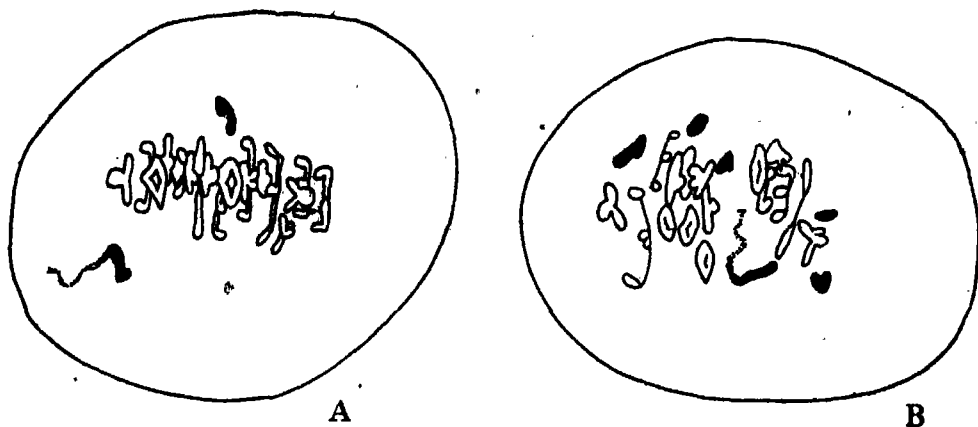


FIGURE 3. Spermatocytes with unpaired sex chromosomes (A) and autosomes (B). $\times 2300$.

Breeding season and age

The natural mating season seems to extend from April to October. Gestation lasts 16 days. In captivity, litters may be borne throughout the year by animals kept warm under healthy conditions. Fertility, however, is much lower out of season and the testes are smaller, particularly in animals over 12 months old. The number of cells in meiosis decreases also, but spermatogenesis is not arrested during the non-breeding season. In this respect hamsters differ from mammals which hibernate during the winter, such as the ferret and the mole (Koller 1936).

Male hamsters are sexually mature as early as $2\frac{1}{2}$ months after birth. In the second breeding season spermatogenesis is greatly reduced; the seminiferous tubules are filled with debris of degenerated cells. Sperm was still present in the testis and epididymis of 24 months old males in June, but the animals failed to mate.

The numbers of spermatocytes showing the starved differential segment are given in table 2 for age and season. Frequencies from brothers are considered together. In the youngest animal analysed, aged $2\frac{1}{2}$ months (number 26), the spermatocytes of neither testis showed any nucleic acid starvation. The 19 bi-valents, including the XY, were packed close together and formed a small equatorial plate. The sex bivalent could not be identified because the differential segment

was not unspecialized. On the other hand, in a 28 months old male (number 14) the majority of spermatocytes showed starvation. It was in this male that spermatocytes with unpaired sex chromosomes and autosomes were found (figure 3 A, B, and plate 5, figure 6).

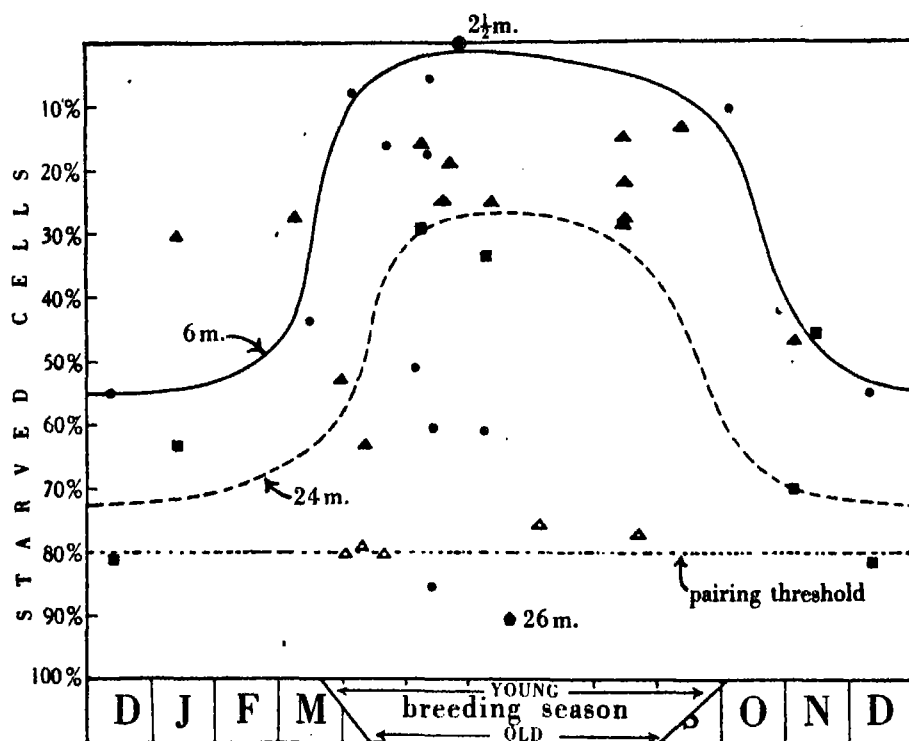
TABLE 2. THE FREQUENCY OF SPERMATOCYTES SHOWING THE DIFFERENTIAL SEGMENT IN AND OUT OF SEASON AT DIFFERENT AGES

number	date of birth	date of fixation	age in months	number of cells	starved		
					number	%	
in season							
26	17. iii. 41	30. v. 41	2½	42	—	—	
21a*	10. i. 41	11. v. 41	4	47	3	6.3	
1a	10. v. 41	5. x. 41	{	53	2	3.8	
2a				5	102	11	10.8
3a				5	47	8	17.2
			total	202	21	10.4	
19b	6. x. 41	13. ix. 42	{	11	29	4	13.8
20b				11	58	8	13.8
			total	87	12	13.8	
8a	5. v. 41	7. v. 42	{	12	50	7	14.0
9a				12	69	13	18.8
			total	119	20	16.8	
8b	5. v. 41	6. v. 43	{	24	47	19	40.4
9b				24	57	12	21.0
			total	104	31	29.8	
10a	10. vi. 41	12. vi. 42	12	27	7	25.9	
10b	10. vi. 41	10. vi. 43	24	26	19	33.8	
14b†	27. iv. 41	21. vi. 43	26	21	19	90.4	
out of season							
21b	10. i. 41	14. i. 42	12	52	16	30.8	
1b	10. v. 41	30. iii. 42	{	11	38	21	55.3
2b				11	39	19	48.7
3b				11	51	29	56.7
			total	128	69	53.8	
19a	6. x. 41	15. iii. 42	{	5	47	17	36.17
20a				5	40	21	52.5
			total	87	38	43.7	
11a	3. xi. 41	4. xi. 42	12	61	29	47.5	
11b		5. xi. 43	24	44	31	70.5	
14a†	27. iv. 41	10. xii. 42	20	47	39	82.9	

* The two testes are called a and b.

† Number 14 failed to mate after he was 18 months old. Unpaired chromosomes were found in the spermatocytes of both testes.

The data in table 2 and in graph 1 show that both age and breeding season are factors which influence the nucleic acid charge of heterochromatin during meiosis. They also influence spermatogenesis in a parallel manner. When spermatogenesis is active, nucleic acid supply is free. When spermatogenesis is restricted, nucleic acid supply is still more restricted and starvation results.



GRAPH 1. The average frequency curves of spermatocytes with the nucleic acid starved differential segment are shown for 6 and 24 months old animals throughout the year. Actual frequencies are plotted. \odot indicates animals from 4 to 8, Δ from 10 to 16, and \square from 20 to 24 months old. Animals kept on normal diet are shown by solid, those kept on reduced diet by clear symbols. The age in months of the youngest and oldest animals is given. The pairing threshold or the failure of chromosome pairing when the frequency of nucleic acid starved cells is above 80 % is indicated.

Diet and disease

The daily diet of the hamsters was composed of (a) rat cubes (supplied by the North Eastern Agricultural Co-operative Society, Aberdeen), (b) nuts, (c) dry biscuits, (d) green stuff or carrots, (e) milk and (f) water. An accurate assessment of the daily food intake was impossible because of the hoarding habit of the animals, but surplus food was usually present in the cages. They were fed daily, except on Sundays when milk and water only were supplied. In winter a few drops of cod-liver oil were added to the food. My experience suggests that milk is an

important item in the diet of hamsters, for when the animals were deprived of it they soon showed signs of distress. Milk presumably supplies deficiencies of light or food in captivity, since it is absent from their diet in nature.

The effects of deficient or restricted diet were investigated in two series of experiments in which the food consisted of rat cubes and water only. In the first series, one testis of a hamster, previously kept on normal diet, was excised and fixed for analysis. Subsequently, such partially castrated animals were put on the restricted diet for various periods. They were then killed and the remaining testis was fixed. In the second series the animals were put on the restricted diet and partially castrated after different periods had elapsed. Then the animals were returned to normal diet for 3-4 months, killed and the second testis was fixed.

TABLE 3. THE EFFECT OF DIFFERENT DIET ON THE FREQUENCY OF SPERMATOCYTES SHOWING THE DIFFERENTIAL SEGMENT

number	date of birth	date of fixation	age in months	number of cells	starved		
					number	%	
normal diet							
6a	19. v. 42	13. xii. 42	7	51	27	55.0	
7a	1. ix. 42	3 iv. 43	7	36	3	8.3	
13a	7. viii. 42	20. iv. 43	8	40	7	17.5	
17b	15. ix. 42	15. viii. 43	11	31	7	22.2	
18b			11	28	8	28.5	
4a	19. v. 42	20. v. 43	12	50	12	19.2	
5a			12	28	3		
15b	10. v. 42	14. viii. 43	15	40	6	15.0	
16b	7. iv. 42	14. viii. 43	16	38	11	28.2	
22a	20. v. 41	12. i. 43	20	47	30	63.8	
reduced diet							
number	date of birth	date of fixation	age in months	number of cells	starved		length of starvation in days
					number	%	
6b†	19. v. 42	19. iv. 43	11	21	17	80.9	119
7b	1. ix. 42	7. vi. 43	9	44	27	61.3	60
13b	7. viii. 42	5. v. 43	8½	45	23	51.1	17
17a	15. ix. 42	14. v. 43	8	23	14	60.8	21
18a			8	20	17	85.0	21
4b	19. v. 42	3. vii. 43	14	38	29	76.3	60
5b		24. viii. 43	15	49	38	77.4	88
15a	10. v. 42	12. iv. 43	11	27	17	62.9	36
16a	7. iv. 42	12. iv. 43	12	29	23	79.0	36
22b*	20. v. 41	3. iii. 43	22	—	—	—	60

* Senile male analysed during the out-of-season period. Meiosis is arrested at pachytene stage.

† Animal was showing signs of serious undernourishment.

The experiments were timed in order to analyse the animals at the peak of the full breeding season. The data obtained are shown in table 3 and graph 1. The simple diet increased the proportion of spermatocytes showing the unspiralized

differential segment. The effect of the deficient diet was superimposed on the variations in nucleic acid charge due to age and breeding season. Moreover, the frequency of spermatocytes showing depletion of the heterochromatin was correlated with the duration of malnutrition. Those animals which were kept longest on reduced diet showed the highest frequency of nucleic acid starved cells. In animals which were kept on the poor diet for 17 days, the number of spermatocytes showing the differential segment was increased from 17.5 to 51.1 %. The longest period spent on this diet by any hamster was 119 days (from December to April). At the end the animal (number 6) was emaciated and had lost considerable weight; the heterochromatin starvation had increased to 80.9 % in spite of the breeding season. In a senile male (number 22), kept on reduced diet for 2 months, spermatogenesis was completely arrested. Meiosis did not proceed beyond the pachytene stage.

TABLE 4. EFFECT OF DISEASE ON THE FREQUENCY OF CELLS SHOWING THE DIFFERENTIAL SEGMENT

number	date of birth	date of fixation	age in months	number of cells	starved	
					number	%
in season						
23a	2. vi. 41	2. iv. 42	10	26	21	80.8
24b	13. ix. 41	10. v. 43	20	23	20	86.9
25a	20. v. 42	21. v. 43	12	47	12	25.5
out of season						
23b	2. vi. 41	3. xi. 42	17	—	—	—
24a	13. ix. 41	21. xii. 42	15	31	16	51.5
25b	20. v. 42	15. xi. 43	18	30	14	46.6

Number 23. Scabies; autopsy revealed pathological abnormalities in the liver.

Number 24. Scabies; developing between *a* and *b* fixation.

Number 25. General debility; cause unknown.

Table 4 contains data obtained from three animals all suffering from disease. Though they were kept on normal diet the intake of daily food was much reduced. There was an increase in the number of spermatocytes showing nucleic acid starvation, but this may have been due partly to undernourishment and not to illness alone. One of the animals (number 23) had an enlarged liver and spermatogenesis was suppressed.

When spermatogenesis is restricted the nucleic acid charge is reduced even more, and apparently its accumulated effects appear in three degrees: (1) nucleic acid starvation, (2) failure of chiasma formation and consequently metaphase pairing, (3) failure of chromosome division and consequent failure to pass beyond the pachytene stage. This last may be due to nucleic acid starvation, but it may be caused by protein starvation since reduplication of chromosomes requires both protein and nucleic acid.

Allocyely of the differential segment

Caspersson (1936), Claude & Potter (1943) and others have shown that the chromosomes are composed of desoxyribose nucleic acid and protein. The maximum nucleic acid charge is at metaphase, the minimum during resting stage. Because nucleic acid is stained with basic dyes, it is assumed to be lacking in chromosomes or chromosome regions which react negatively to these stains. Under the influence of various factors, particular chromosome regions show differential reactivity in respect of the nucleic acid which is attached and detached at different stages of division and at different rates from the normal. This behaviour, called 'allocyely' (Darlington 1941), is a characteristic property of the heterochromatin and distinguishes it from euchromatin regions which are regular and consistent in their nucleic acid cycle (Pontecorvo 1944). Allocyely of the differential segment of the sex chromosome of the hamster during meiosis is shown both by excess charge at prophase and deficient charge at metaphase.

The differential reactivity of heterochromatin in the chromosomes of plants and animals can be shown by cold treatment. In the chromosomes of animals, food starvation has been found to reveal such segments (Wickbom 1945). In the hamster I have now found that not only nutrition (affected by diet and disease) but two other factors, i.e. age and breeding season, play a role in the nucleic acid metabolism and are responsible for the statistical variation in the nucleic acid charge of the heterochromatin in spermatocytes. It seems that whatever causes reduce spermatogenesis likewise reduce even more proportionately the formation of the nucleic acid necessary for spermatogenesis. The supply of nucleic acid or its precursors is, as it were, the dominating variable.

A competition for the nucleic acid or for its precursors was seen to be in operation between euchromatin and heterochromatin of the same cell in *Trillium* and *Triton*. In addition, in *Cricetus* it operates also between different cells adjacent to each other (plate 5, figure 5). What differences between the individual cells determine their success or failure in this competition? The obvious possibility lies in their position in relation to blood supply (Caspersson & Santesson 1942). But no evidence for this was obtained. There is a variety of evidence, such as synchronous division of pollen grains (Barber 1941, 1942), spermatocytes (White 1935) and tumour cells (Koller 1943), which shows that adjacent cells can influence each other and can collaborate with each other in growth and in the nucleic acid supply necessary for growth. In pollen mother cells and spermatocytes this collaboration always seems to work for uniformity. Differentiation and its concomitant competition are unknown. It seems necessary to suppose that the delicate balance of supply and demand in regard to the nucleic acid charge of the heterochromatin in the hamster reveals differences between cells which are concealed with any other discriminating test.

It is known that during rapid growth the demand for protein and nucleic acid is extremely great. In the fertilized eggs of plants and animals this demand is met

by a very high concentration of ribose nucleic acid, the conversion of which into desoxyribose maintains rapid cell multiplication (Caspersson 1936, 1937; Brachet 1940; Painter 1943). Similarly, in the young hamsters the supply seems to be in excess; it not only satisfies the demand but even leaves a margin to spare which makes the nucleic acid charge independent of external disturbances. I found nucleic acid starvation of the heterochromatin in young hamsters under abnormal, as well as normal, conditions to be negligible. If the thymus gland, which is present only in young animals, has some influence on the nucleic acid metabolism during growth, this could now be tested experimentally.

The rate at which nucleic acid metabolism is affected will be different for different tissues. In *Trillium*, after only 30 min. exposure at 0° C, nucleic acid starvation is said to be shown within the cell (Wilson & Boothroyd 1941). In the liver cells of rats, Kosterlitz (1944) reported that 1 day on protein-free diet leads to 15 % reduction in the content of nucleic acid and protein. This reduction was shown by Davidson & Waymouth (1944) to be due to a fall in the amount of ribose nucleic acid in the cytoplasm. The first effect of reduced diet on the heterochromatin in the spermatocytes of hamsters was observed after 17 days.

There are other questions apart from the four factors analysed which bear on the problem of nucleic acid starvation in the hamster. Of these the following are to be investigated. First, so far it is not known how much the decreased nucleic acid supply in the winter is due to the change in feeding and to the season. Secondly, it is not known what is the cause of the yearly variation which is indicated by comparison of data from 1942 to 1943. Thirdly, nothing is known of the special vitamin requirements of the hamster. Deficiency of specific vitamins rather than reduced diet itself might be the primary factor in reducing the nucleic acid metabolism. And finally, there are evidently genetic differences between strains which affect nucleic acid starvation. These additional factors cannot be identified by our experimental methods at present.

Chromosome behaviour and nucleic acid supply

In the hamster, extreme nucleic acid starvation of the heterochromatin is frequently associated with a failure of pairing of the sex chromosomes and occasionally that of the autosomes. Now it is known that post-pachytene pairing of chromosomes depends on chiasma formation which in turn depends on chromosome reduplication. It seems that there is a threshold within the cell, and when the amount of nucleic acid falls below it, gene and chromosome reduplication is delayed and crossing-over will be prevented (graph 1). Thus the failure of metaphase pairing in cells with starved heterochromatin may well be due to the reduced nucleic acid supply. If the reduction is extreme, development of spermatocytes beyond the pachytene stage is arrested.

Indirect nucleic acid starvation of heterochromatin due to age, season and diet is never uniform in the cells of a particular tissue in animals. Intra-chromosomal and especially inter-cell differences were found to be much greater in the spermato-

cytes of hamster than in the root-tip cells of *Paris*. It seems likely that while nucleic acid starvation in *Paris* depends on the failure of conversion of desoxyribose from ribose nucleic acid, in the hamster starvation of heterochromatin depends on the restriction of the basic store of ribose nucleic acid in the cytoplasm. The inter-cell differences as seen in the hamster would then be due to differences in the distribution of ribose nucleic acid or its precursors in the tissue. The starvation caused by cold, on the other hand, is invariable owing to the constant effect of temperature on a relatively limited and precise series of chemical reactions. The process responsible for the arrest of cell division by ionizing radiations is assumed to be a similar reaction but of much greater intensity than that induced by cold treatment. It has been suggested that the reduction of ribose to desoxyribose nucleic acid is prevented by X-rays (Mitchell 1942).

The heterochromatin, on account of its specific structure and reactivity, is a regulator of the nucleic acid metabolism during division and resting stage. It is responsible for maintaining equilibrium between demand and supply. This balance may be upset by external factors (temperature, nourishment), or by internal factors such as the addition of an extra heterochromatic chromosome to the normal chromosome complex. It is known that such an addition is followed by an increase in the ribose nucleic acid content in the cytoplasm, e.g. in the *XXY* egg of *Drosophila* (Caspersson & Schultz 1939) and leads to supernumerary division in *Sorghum* (Darlington & Thomas 1941). Furthermore, increased nucleic acid content and increased rate of division in tumour tissue were shown to be connected (Caspersson & Santesson 1942; Koller 1943). It seems, therefore, that abnormal chromosome behaviour and abnormal mitotic cycle can be attributed to a common cause, namely, to an excessive nucleic acid supply. Differences in nucleic acid supply also seem to be responsible for the cell differentiation in the development of blood in the bone marrow (La Cour 1945). By studying the physiological control of cyto-chemical starvation, therefore, some light may be thrown on the abnormalities of differentiation in the bone marrow which are associated with various types of blood disease.

The differential reactivity of the heterochromatin in the sex chromosome of the hamster and the abnormalities correlated with it under variable conditions provide yet another means of testing the functions of nucleic acid in the activity of the cell.

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DESCRIPTION OF PLATE 5

FIGURE 4. First metaphase of meiosis showing the unspiralized differential segment of the asymmetrical sex bivalent. $\times 2300$:

FIGURE 5. First metaphase of meiosis in two spermatocytes; in one the differential segment of the X-bivalent is starved of nucleic acid, in the other it is fully charged and therefore cannot be identified. $\times 2300$.

FIGURE 6. Meiosis in four spermatocytes. In the third from the left the sex chromosomes and some of the autosomes are unpaired. The unspiralized differential segment of the X-chromosome is visible. $\times 2000$.

The fine structure of the wall of the conifer tracheid

I. The X-ray diagram of conifer wood

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[Plates 6, 7]

X-ray diagrams of the wood of a number of species of conifers are examined in the light of the geometrical principles underlying the spiral diagram. It is shown that the meridional arcs in the diagrams, which have been reported to represent transversely oriented cellulose chains in the tracheid wall, are spurious; they arise mainly from overlapping of the ends of the lateral arcs and a fusion with this overlapping region of arcs with nearly the same spacing. It is also suggested that the ray tissue contributes towards the meridional arcs.

Measurements of layer thickness in the tracheid wall and of the double refraction of the outer layer in the tracheid, show that there is no correlation between the properties of the outer layer and the appearance of meridional arcs. On the contrary, this evidence suggests very strongly that the outer layer does not differ from the central layer in cellulose chain direction, and that the wall is therefore uniform in this respect. It is considered that the marked optical and swelling heterogeneity in the tracheid wall is therefore to be sought in variations of some property other than cellulose chain direction, and it is again pointed out that changes in angular dispersion of the cellulose matrix offer a possible solution.

INTRODUCTION

One of the more interesting features in cell-wall studies during recent years is the occurrence in a number of cell types of what has come to be called the 'crossed fibrillar' structure (Frey-Wyssling 1938), i.e. the presence in a wall of layers with different cellulose chain directions, each layer having the same direction as the last layer but one. This has been proved to occur in *Valonia* (Preston & Astbury 1937), *Chaetomorpha* (Nicolai & Frey-Wyssling 1938) and *Cladophora* (Astbury & Preston 1940) among the algae, in some vessels, though not all, in higher plants (Preston 1939*a*), and to a less extensive degree in cotton hairs (Anderson & Kerr 1938). There is no doubt that this type of structure confers upon a cell very special properties and presents besides a problem of the first magnitude in the question of the mechanism by which the wall layers are deposited. It is therefore a matter of some importance to define the conditions under which 'crossed fibrillar' structure is observed; and an investigation of the frequency with which it occurs in the many cell types offering themselves for consideration provides one line along which information on this point might be sought. To this end, the investigator must always be careful to substantiate completely the presence of this structure, and this is particularly desirable when working with materials whose properties are of academic or commercial importance when these properties are to be interpreted in terms of wall structure.

On the basis of optical properties and associated swelling phenomena this general type of structure has been suggested for some of the elongated cells of the higher plant, notably phloem fibres and the xylem tracheids of conifers. This type of evidence has already been dealt with (Preston 1939*a*, 1941; Majumdar & Preston 1941; Kundu & Preston 1940), and, in brief, it may be said that the following points have emerged:

(1) The optical properties concerned are capable of various interpretations, and it is dangerous without corroborative evidence to choose any one in preference to the others.

(2) Swelling of elongated cells can and does produce profound changes in cellulose chain direction, so that observations on swollen material should be interpreted with caution. The gross displacements which must occur if crystals (even though visible only under the microscope) are grown in the wall may be included here.

(3) The various wall layers differing in optical properties vary also in swelling behaviour as the 'crossed fibrillar' hypothesis is said (probably incorrectly) to demand; but similar variations of swelling also occur between layers of the same optical character, so that the connexion between optical properties and swelling behaviour is probably quite fortuitous. Further, this differential swelling causes the appearance of apparent transverse striations in the non-swelling layer (often the outermost layer of the wall). These are actually folds, and not striations proper, depending solely on the spatial relations of the swelling layer and reflecting nothing therefore of the fine structure of the layers in which they occur.

(4) The optical heterogeneity of some at least of these cell types might be explained by changes in the *angular dispersion** of the cellulose chains with no change in net direction. With phloem fibres (Kundu & Preston 1940; Preston 1941) and collenchyma (Majumdar & Preston 1941) the case is perfectly clear. The X-ray diagram, both of bundles of cells and of individual fibres, fails to show any sign of chain orientation other than the longitudinal or steeply spiral. Any layer with chains oriented transversely must be tenuous in the extreme—certainly thinner than the layer which appears bright in cross-section under the polarizing microscope.

The corresponding condition in the elements of the xylem has been investigated by Bailey and his co-workers (Kerr & Bailey 1934; Bailey & Kerr 1935; Bailey & Vestal 1937). In transverse section a conifer tracheid wall is three-ply at least. In general, a thin outer layer and an even narrower innermost layer are bright in transverse section between crossed nicols and a central layer, much thicker in late- than in early-wood tracheids, is darker (cf. plates 6 and 7, figures 8, 10, 15 and 17). From this and other evidence, Bailey concludes that the cellulose chains in the inner and outer layers run in flat spirals, while those of the central layer are tilted in a steep spiral. The presence of flat spirals has been contested by the writer (1939*c*) following

* For explanation of this term, see p. 331.

an earlier statement (1934) that in the tracheid wall there occur chains running in one direction only. As a result of a reinvestigation it was proposed that the optical heterogeneity is due to changes in angular dispersion, and a model was proposed which Bailey & Berkeley (1942) have apparently completely misunderstood. In the last analysis the evidence for a 'single fibrillar' structure here rests largely upon the X-ray diagrams of single-conifer tracheids, only one of which has been published (Preston 1934), and this has now been criticized in a paper (Bailey & Berkeley 1942) in which further X-ray work is presented. While this criticism of the published X-ray diagram of a single tracheid in no way invalidates a considerable body of other evidence upon which the 'single fibrillar' hypothesis is based, the further X-ray diagrams presented by Bailey & Berkeley must be considered in some detail, and it is the purpose of the present paper to re-examine this later work.

The absence in the X-ray microphotograph of any arcs corresponding to transverse chains Bailey & Berkeley dispose of with a facile dismissal of the whole photograph, since 'neither this photograph nor its enlargement exhibit a clearly defined diffraction pattern'. They fail, however, to remark either upon the very cogent arguments which make it imperative to use photographs of *single* cells in this work, or upon the difficulties involved in obtaining such photographs at all and the great loss of clarity in reproduction. In the original photograph the absence of the arcs in question is undoubted—otherwise the photograph would not have been published—and any interpretation of wall structure which ignores this point is bound to be worthless. It is quite clear that the presence or absence of transverse chains can be proved directly only in such diagrams of single tracheids. In even a small chip of wood there are hundreds or thousands of tracheids each with cellulose chains running in a spiral whose angle differs from cell to cell, each giving X-ray diagrams of a very complex type; in the radial wall, at least, of each tracheid there are numerous bordered pits in which the cellulose chains run almost in circles (Preston 1939a); and finally there is the ray tissue which in some cases (Gross, Clark & Ritter 1939) can give diagrams of its own. The X-ray diagram even of such a simple tissue as conifer wood is therefore very complex, and it is for this reason that the writer originally undertook the laborious manufacture of an X-ray microspectrometer and the presentation of a diagram of a single tracheid, and to take the added precaution of flattening the cell completely to avoid the complexity inherent in the spiral. In an attempt to discredit such photographs, Bailey & Berkeley present a series of photographs of pieces of wood 1 mm. thick and, in spite of their own warning as to the dangers involved, make an attempt to show from this composite material the presence, in a single layer of a single tracheid, of cellulose chains running in a flat spiral. Such photographs involve, of necessity, all the complications which the microspectrometer was designed to avoid, and their interpretation is therefore to be approached with extreme caution.

The main point at issue in these newer photographs is that, besides the principal lateral arcs or spots which certainly correspond to a steep spiral (plate 6, figure 11),

there occur along the central vertical line of the diagram (called the meridian) two fainter arcs at the same distance from the centre, and these are said to correspond to transverse cellulose chains. Confining attention for the moment to the outermost arcs, these correspond to molecular planes spaced 3.9 Å apart and lying parallel to the cellulose-chain direction. Within them, in every case, may be seen two other arcs (often fused) corresponding to planes also lying parallel to this direction but spaced 5.4 and 6.1 Å apart. Since the tracheids lie parallel to the meridian, then each radial line of arcs may be regarded as lying perpendicular to the corresponding set of cellulose chains. The steeper and less steep sets of chains, whose presence is in this way suggested, are said by Bailey & Berkeley to correspond further to two sets of striations visible in the wall; but since no attempt is made to give a quantitative comparison of chain direction and striation direction, and since the reliability of the latter can be assessed only on the exact correspondence which might then be demonstrated, this adds nothing new, and the whole interpretation stands or falls by the validity of the interpretation of the X-ray diagram. The diagram itself, of course, tells nothing concerning the location in the wood specimen of any oriented cellulose chains whose presence it reveals.

In the previous work of the writer (Preston 1934) no striations of any kind were visible under the conditions used, and no quotation out of context can controvert this; and in view of the dependence of the interpretation of striations on the X-ray diagram, only the latter will be considered here. It obviously demands much more careful attention than has hitherto been given. In the following pages, therefore, these diagrams will be considered in the light of the geometrical principles underlying the spiral diagram. It will be shown that the presence of meridional arcs is completely misleading and corresponds to no specific structure in the wall. Further evidence will also be put forward which indicates that the interpretation put upon the diagram by Bailey & Berkeley is completely erroneous.

THE SPIRAL DIAGRAM

In view of the complexity which may be revealed in the diagram of fibrous material, even of fibres of comparatively simple organization, it will be well first to consider the conditions under which sets of planes in fibres can contribute spots or arcs in any particular position on the photographic plate. The interpretation of subsequent X-ray diagrams will then be simplified. The most convenient way of looking at the evolution of the fibre diagram is with the aid of spherical projection and the pole figure (figure 1). A crystal is imagined as lying at the centre O of a sphere, and the point at which the normal to any set of molecular planes intersects the spherical surface is called the *pole*, P , of these planes. The conditions of reflexion of X-rays from the set of planes are defined by the reflexion circle, $PQRS$, the locus of the pole when the planes are inclined at the glancing angle θ to the X-ray beam. In figure 1 the pole P is drawn in a position for reflexion. The reflecting positions are thus given by the points at which the reflexion circle intersects

the locus of the pole. If the crystal is rotated about Mm the locus of the pole is two small circles L_1l_1 and L_2l_2 , which therefore intersect the reflexion circles at four points P, Q, R and S corresponding to four symmetrically disposed spots P', Q', R' and S' on the photographic plate. It will simplify matters considerably if only those planes of most importance in the present paper are considered, i.e. planes parallel to the direction of rotation. There are then only two positions for reflexion, lying along the equator of the plate and equidistant from the centre (figure 2a).

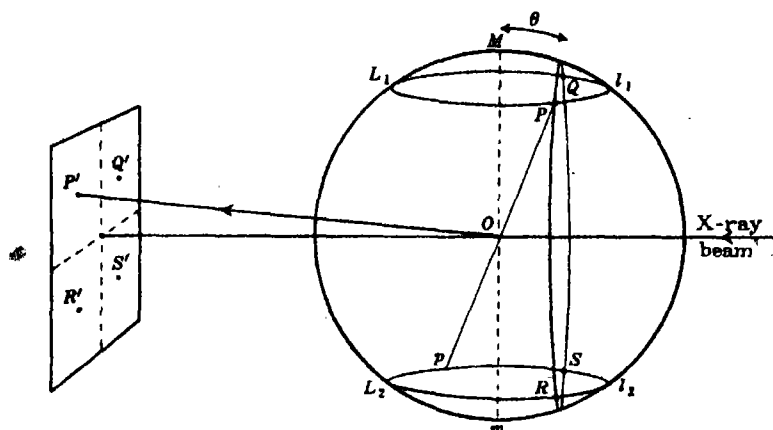


FIGURE 1

If now the crystal is replaced by a bundle of parallel fibres, in which the cellulose chains run longitudinally, arranged to lie parallel to the rotation axis Mm , then figure 2a gives also the derivation of diffraction spots from planes lying parallel to the cellulose chains. The major difference between this ideal geometrical pattern and that of a real natural fibre is merely that no part of the fibre represents a real perfect crystal. Its cellulose component consists of innumerable minute regions—the micelles—in which the chains are strictly parallel and arranged in a regular manner, separated by 'intermicellar' spaces in which the chains run from micelle to micelle in a random fashion. These micelles are elongated and on an average lie parallel to the length of the fibre; they are, however, tilted to a greater or less degree to this common line of orientation, i.e. they have *angular dispersion*, and this can be allowed for by allowing the axis Mm to wobble slightly as it rotates. The intersection of the axis with the projection circle then describes two polar caps limited by two small circles MM' and mm' , and the locus of the pole is a family of circles of which two are represented in figure 2b; the spots are therefore drawn out into two arcs PP, QQ , which are more intense at their centres.

Now to derive from this construction the pattern to be expected from a set of planes parallel to the cellulose chain direction in a fibre wound with a spiral, one has merely to remember, that the spiral is formed in effect by a tilt of the micelles through a constant angle to the longitudinal, in the plane of the fibre wall. Taking

any small element of the wall, therefore, the axis of rotation is tilted through some angle S , and the effect may be seen in figure 2b if α is replaced by S in the diagram. The circle Ll , the locus of the pole to which Mm is normal, is tilted through the same angle S , and its intersection with the reflexion circle is therefore depressed.

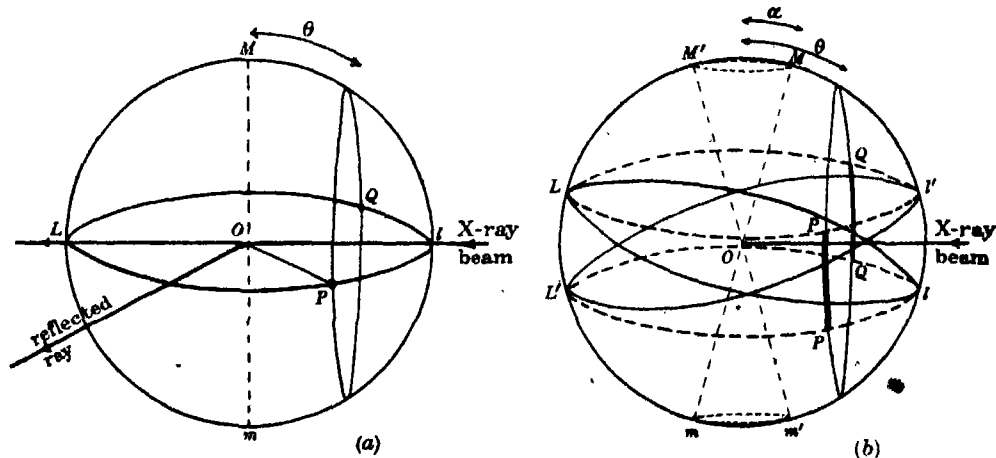


FIGURE 2

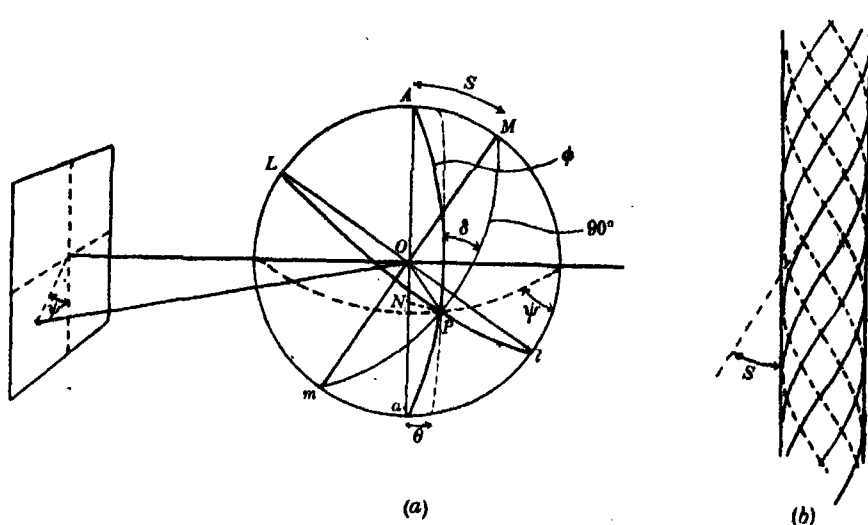


FIGURE 3

To represent the whole figure the axis Mm is rotated about the direction of the fibre length, the axis traces out the hollow cone $MmOM'm'$, and the locus of the pole P becomes the belt $LlL'l'$ bounded by two broken small circles in figure 2b. The lateral spots are again therefore drawn out into two arcs whose lengths depend on the value of the angle S of the spiral. Clearly, however, the intensity distribution along the arcs will be different from that referred to in the arcs derived above

for angular dispersion. The cone $MmOM'm'$ is now hollow instead of solid, and it is no longer evident that the centre point of each arc will have the highest intensity. On the contrary, it can be shown that the intensity is now highest at the ends of the arcs; this may be seen quite simply in the following way.* In figure 3a let S be the spiral angle (cp. figure 3b), ϕ the angular distance of the pole at P from the spiral axis Aa , δ the angle between the great circles MPm and APa , and PN the perpendicular from P to the spiral axis. Then since the great circle Ll is a line of equal pole density, the density at P on the surface of the sphere is inversely proportional to PN and $\sin \delta$, i.e.

$$D_P \propto 1/(\sin \phi \sin \delta),$$

but

$$\sin \delta = \sqrt{(1 - \cos^2 S / \sin^2 \phi)},$$

therefore

$$D_P \propto 1/\sqrt{(\sin^2 \phi - \cos^2 S)}.$$

The corresponding intensity in the photograph, I_P , is proportional to D_P , but ϕ must be converted into a vector measurable on the photograph. This can be done by substituting

$$\cos \phi = \cos \theta \cos \psi.$$

Hence

$$I_P \propto 1/\sqrt{(1 - \cos^2 \theta \cos^2 \psi - \cos^2 S)}.$$

I_P is therefore a maximum at the points

$$\psi = \cos^{-1} (\sin S / \cos \theta), \quad 180 - \cos^{-1} (\sin S / \cos \theta),$$

since at these points the intensity is theoretically infinite. This means that the intensity is the greatest at the ends of the arcs. Each arc therefore breaks up into two spots and the diagram consists of four spots, symmetrically placed, instead of two lateral arcs. If to this be added the angular dispersion of the micelles it is seen that the spiral photograph is, indeed, to quote Astbury's own words, 'a versatile trap for the unwary'. Depending on the value of S , the four arcs may overlap on the equator, giving a false impression of a real equatorial spot, or even, if the dispersion is great enough, fuse into two meridional arcs. Finally, overlap may occur in both positions, giving eight apparent arcs in all, only four of which are real. Several further points appear from the equation for intensity:

(1) Providing the dispersion is not unduly high, a fibre of circular cross-section gives a photograph in which the expected four arcs appear symmetrically placed. There is therefore no point in attempting to connect the appearance of four arcs with flat-sided cells as Bailey & Berkeley have done.

(2) As S , the spiral angle, increases, the four spots approach the meridian more and more closely. As this happens, the danger of an overlapping of these

* The geometrical relationships of the general plane, from which the following special case is derived, have been worked out by Dr W. T. Astbury, F.R.S., for whose permission to use this derivation the writer is deeply indebted.

arcs along the meridian, giving a spurious arc, becomes greater. At a value of S given by $\cos S = \sin \theta$, the spots fuse into two meridional arcs (because at this value of S , $I_P = \infty$ only when $\psi = 0$ or 180°). Taking the planes of 3.9, 5.4 and 6.1 Å spacing, the critical values for S appear to be as follows:

spacing	S (limiting)	
	no dispersion	dispersion $\pm 5^\circ$
3.9	78.5°	73.5°
5.4	81.8°	76.5°
6.1	82.8°	77.5°

Hence all fibres whose spirals are flatter than $S = 73.5$, say, for the 3.9 arc, or than the corresponding figure for the other two arcs, will give two meridional spots instead of four lateral arcs. In a mixture of fibres in which S varies sufficiently widely, therefore, one would expect those spirals flatter than those given in the above table to contribute to the meridional arc, and the 3.9 arc should therefore appear intense out of proportion to the 5.4 and 6.1 arcs. It is interesting to note that this is the case here, and that in tracheids generally spirals do occur with sufficiently great values of S (Preston 1934).

(3) If the spiral is flat, i.e. $S = 90^\circ$, then the intensity equation reduces to

$$I_P \propto 1/\sqrt{1 - \cos^2 \theta \cos^2 \psi}.$$

At $\psi = 0$, therefore, the intensity is proportional to $1/\sin \theta$, and at $\psi = 90^\circ$ it is proportional to unity. One has, then, the arcs drawn out into a continuous circle with the intensity rather greater along the meridian. Looking at it another way, a set of chains running round a tracheid in transverse circles or a very flat spiral, would give two very wide arcs in the meridional position whose arms would meet at the equator to give a complete ring. In obvious distinction from such wide arcs are the rather sharp meridional arcs observed by Bailey & Berkeley and those presented in the present paper.

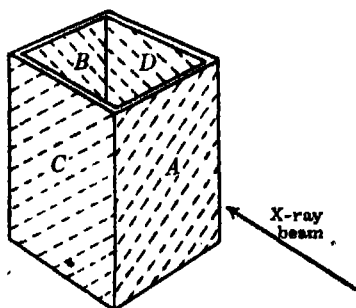


FIGURE 4

Therefore it is seen that in the diagram given by a bundle of fibrous cells with spiral cellulose chains four major arcs may be expected from the planes of spacing 3.9 Å, and four minor arcs, two along the equator and two along the meridian. For the sake of completeness it may also be noted that equatorial arcs may be

derived in another way. Suppose the tracheids are rectangular in cross-section, with two opposite faces normal to the beam (figure 4). Then the walls *A* and *B* will clearly give four major arcs in diagonal positions and perhaps spurious equatorial or meridional arcs or both. The walls *C* and *D*, however, will give arcs which are nearly equatorial. Their actual positions may be determined in the following way. Figure 5 shows the projection circle and pole figure appropriate to this case, the symbols having the meanings common to this paper. The relation

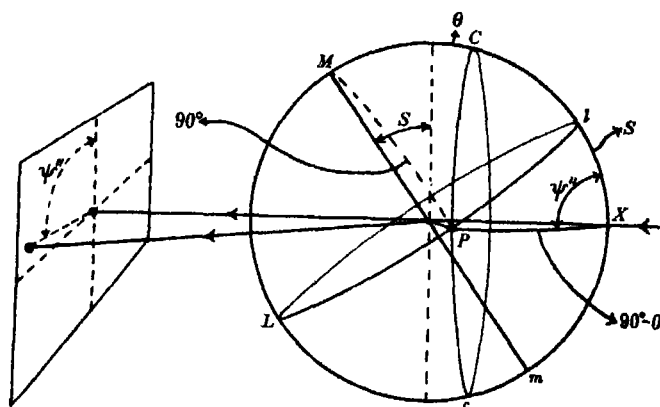


FIGURE 5

$\cos \psi'' = \tan S \tan \theta$ is obtained from the spherical triangle MPX . For the arcs given by the walls A and B normal to the beam the angular distance of each from the meridian is $\psi^L = S$. One has to compare ψ'' with ψ^L . This can be done with specific examples as in the following table for the planes of spacing 3.9 Å:

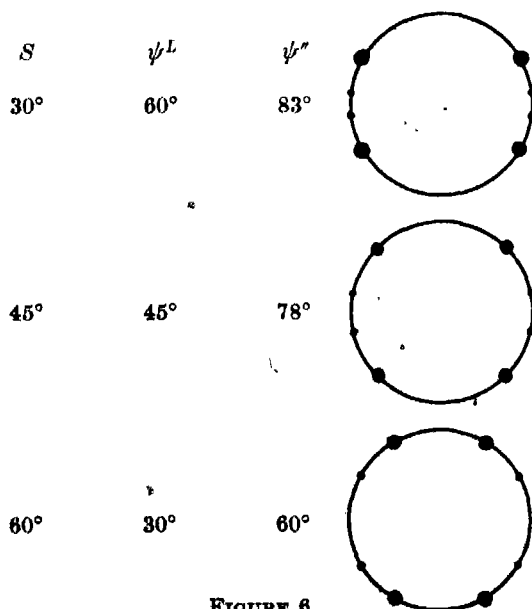


FIGURE 8

The positions of the arcs are shown graphically in figure 6. Even with a spiral as steep as $S = 30^\circ$ the arcs corresponding to the lateral walls are displaced by 7° from the equator and therefore separated by 14° . Sufficient dispersion would cause these arcs to overlap along the equator, but these fused arcs would then of necessity be very diffuse, ranging over an angular distance of the order of 35° . Except, therefore, for the steeper spirals one should not expect a sharp equatorial arc from the lateral walls; and in these steeper spirals an arc of this type would probably be masked by the arcs from walls *A* and *B* which then approach the equator. The few equatorial arcs observed by Bailey & Berkeley and in the present work seem too sharp to be explained along these lines and this is a feature which Bailey & Berkeley appear to have overlooked.

Finally, the greatest care must be taken, in the interpretation of these diagrams in which the 3·9 arcs, and therefore the whole photograph of the set of chains, is tilted from the normal position, to observe the possible fusion of arcs derived from other planes. These fusions, which would normally occur along the equator or along the meridian, might be placed sufficiently near the 3·9 arcs, real or spurious, so that these then become unduly pronounced. While these would usually be distinguishable from true 3·9 arcs on account of their different spacing, there are planes in the cellulose structure sufficiently near the 3·9A spacing to cause confusion particularly if they come to lie near a 3·9 arc or even fuse with it. It will be seen that this is a not inconsiderable factor in the present case.

There are thus at least four ways in which spurious meridional arcs may be produced, and when it is remembered that in actual specimens the ray tissue may also add to their appearance, then it becomes abundantly clear that much careful scrutiny of diagrams is needed before pronouncement can be made on the reality of these 'abnormal' spots.

METHODS AND RESULTS

A. General

A brief survey of the diagrams presented by Bailey & Berkeley in the light of the last section gives a number of pointers towards the further work which is necessary to determine the underlying features of structure manifested in the meridional arcs. Starting from the self-evident fact that an arc which is not present in the diagram of a single flattened tracheid cannot possibly appear in that of a bundle of tracheids unless it is derived from the spiral form of the chains in the unflattened cells, from an interaction between the diagrams of individual tracheids in the bundle, or from some source outside the tracheids, it is interesting, first to notice that Bailey & Berkeley present only one photograph of a wood specimen in which the meridional arc is missing, and only in this specimen do the cellulose chains run almost exactly parallel to the tracheid length, i.e. the spirals are steep and there is little variation in spiral angle from cell to cell. Under these

conditions one would expect little danger from overlapping of arcs along the meridian. On the other hand, the meridional arcs are strong in the diagrams of those specimens whose component cellulose chains run in flatter spirals, and it may be said that in general these arcs become stronger the flatter the spiral and the greater the angular dispersion. This appearance of the arcs the authors attempt to correlate with the thickness of the outer layer of tracheid walls in relation to that of the central layer as seen in transverse section under the polarizing microscope. The actual dimensions, vitally important as they are in such a connexion, are not given, but, judging from the photomicrographs which accompany the diagrams, it is clear that the variation in layer thickness is not nearly enough to account for the variation in intensity of the meridional arcs. This point will be examined later with the help of actual figures for layer thickness. There is, then, already some evidence that the appearance of the meridional arc has something to do with the flattening of the spiral in the wall and a suggestion that the overlapping of arcs is involved. Closer study shows that the distance of the outer meridional arcs, for example, from the centre of the photograph corresponds to a spacing rather larger than the 3.9A to which the lateral arcs correspond exactly. This is particularly clear in the photograph of *Sequoia sempervirens* (Bailey & Berkeley 1942, figure 17). In turn, this suggests that part of the overlap along the meridian consists of a fusion of arcs other than the 3.9 with the 3.9A arcs themselves, and these are probably the 0.21 arcs (see plate 6, figure 9 of this paper). In some of the diagrams this inner arc is just visible as a separate unit inside the true 3.9A arc. The intensity of the 3.9A meridional arc is thus visually over-emphasized both on this account and because (as the work to be described here strongly suggests) the photographs are in any case badly over-exposed.

With these points in mind it is proposed here to follow the flattening of the spiral in the tracheid wall from the steep spirals of some wood species through spirals of medium slope to the flattest spirals, in order to study the overlapping and fusion of arcs as the lateral arcs become more and more tilted from their equatorial positions; and to study the same range in a suitable single species of wood by passing from outer to inner annual rings of the same cross-section of a tree trunk (where, it is known, the spirals will gradually flatten (Preston 1934; Misra 1939)). At the same time such necessary subsidiary measurements as layer thickness will be made on the parts of the specimens actually photographed in order to check on the variability of these properties. The whole of the work is confined to late wood where the optical heterogeneity is most marked. Wood has therefore been examined of the following species: *Pinus resinosa* Ait. and *P. insignis* Doug.; *Picea Abies* Karst., *Juniperus virginiana* L., *Larix leptolepis* Murr. and *Pseudotsuga Douglasii* Carr, and only straight-grained samples were used throughout. Specimens were also examined of *Abies nobilis* Lindl., *Chamaecyparis thyoides* B. S. & P., *Taxodium distichum* (L.) Rich. and *Sequoia* sp., but, since these add nothing new to the observations made on the former group of specimens, no further reference will be made to them.

For observation on the spectrometer, strips of wood were prepared whose lengths ran exactly parallel to the grain and with two long faces tangential and two radial. The radial thickness of the wood was 1 mm., unless otherwise stated, and the X-ray beam, collimated by a slit 0.5 mm. diameter, ran radially through the specimen. The volume of wood subjected to X-rays was therefore approximately 0.2 mm.³. Each strip of wood was subsequently sectioned and layer thickness determined in ordinary light, using a Leitz Okularschraubenmikrometer under a 1/12 in. oil-immersion lens. For reasons which will become clear, the double refraction of the bright outer layer was also determined with light of a standard wave-length using sections as near 5μ as possible and with the aid of a de Sénarmont compensator. In this latter determination measurements were made on at least two sections as a check on accuracy.

B. The X-ray diagram

The X-ray diagrams presented in plates 6 and 7 offer so striking a confirmation of the view presented above that little further need be said. In *Picea Abies* (plate 6, figure 9) the lateral arcs are comparatively little dispersed, and the cellulose chains in the wall run therefore in very steep spirals. Obviously here no overlapping occurs of the two equatorial arcs on the meridian, and the two 021 arcs are well separated and therefore cause no confusion. Correspondingly, the meridional arcs are missing, in agreement with a similar photograph presented by Bailey & Berkeley for *Pinus longiflora* (1942, figure 7), where a similar condition obviously obtains. In *Pinus resinosa* (plate 6, figure 11) the lateral arcs are longer, corresponding to less steep spirals in the wall, and the 021 arcs are also spread. Here, therefore, a faint meridional arc is evident. It is not clear, however, in this particular specimen whether the overlapping of the lateral arcs can be sufficient to cause this appearance of a meridional arc. The question of the reality of this (very faint) arc will be discussed later on. Passing on through *Larix* (plate 7, figure 18) to *Pinus insignis* (plate 7, figure 16), however, it is seen that as the lateral arcs spread, due to a flattening of the spiral, they overlap more and more on the meridian, and this, together with a fusion of the 021 arcs (still separate in *Larix* but fused in *Pinus*) as they approach each other accompanies the appearance of pronounced meridional arcs. This process has gone still further in *Juniperus* (plate 7, figure 19); in this species, the spiral winding has become so flat that the lateral arcs now show the greatest intensity at the two ends as we expect in a spiral diagram when the angular dispersion is sufficiently small. Here the meridional arc has become very prominent. Finally, in *Pseudotsuga* (3rd annual ring) (plate 6, figure 12) the cellulose chains occur in so flat a spiral that the arcs are spread into an almost complete ring with comparatively little difference between the intensities at the equator and the meridian. Similarly, in passing from the 11th annual ring in *Pseudotsuga* (plate 6, figure 14) through the fourth (plate 6, figure 13) to the third (plate 6, figure 12), a gradual increase is again seen in the spread of the lateral arcs, a gradual fusion of the 021 arcs and a corresponding increase in the intensity of the

meridional arcs from invisibility to very pronounced development. In one and the same section of a woody stem, therefore, a gradual flattening of the spiral is accompanied by the appearance of meridional arcs.

There is thus a close connexion between the inclination of the molecular spiral in the wall and the appearance of meridional arcs in the X-ray diagram, and it seems highly probable that these meridional arcs are spurious and involve merely the overlapping of the ends of the lateral arcs enhanced by the simultaneous fusion of the 021 arcs along the meridional line. With regard to the special case of *Pinus resinosa*, where a faint meridional arc occurs under conditions in which it is difficult to assess the importance of overlapping, it will be shown later that even here the arc in question cannot arise from a secondary wall layer. It remains to decide whether the arc arises from some source in the wood outside the walls of tracheids. The obvious tissue in which first to seek for this source is the ray tissue (the primary wall is too tenuous to treat by the methods used in the present paper; its condition has been examined by different methods and the results will be reported later in a separate paper). It seems quite possible, since rays are known to give X-ray diagrams of their own (Gross *et al.* 1939), that ray tissue of the type found in conifers, where the walls lying transverse in the stem are usually the only ones (if any) to be flattened, then these transverse flattened walls could give meridional arcs in a diagram in which the X-ray beam is oriented at right angles to the grain of the wood. To test this possibility, a tangential longitudinal section of *P. resinosa* was prepared, sufficiently thin that, when mounted on the spectrometer slit, examination under the microscope would show whether or not the beam would impinge on ray tissue. In order to save time of exposure, the section was made only just thin enough for this purpose, i.e. about 300μ . The slit used was rather less than 0.1 mm. in diameter, and the specimen-plate distance was reduced from 3 to 2 cm. Even then the exposure time was 150 hr. and the centre of the photograph became badly fogged. The section was first so adjusted that a beam of light passing through the slit was clear of ray tissue and the corresponding X-ray diagram obtained. The section was then moved until a neighbouring ray occupied the centre of the beam. The two diagrams are presented in plate 7, figures 20 and 21. In spite of the equal exposure times the diagram of the section including a ray appears at first sight more intense than that excluding the ray; this is a consequence of the greater spread of the lateral arcs in the latter diagram and should not therefore affect the appearance of any true meridional arc. Close inspection of the diagrams shows that the meridional arc is more intense in the diagram including the ray than it is in the other. There is therefore some evidence here that ray tissue, too, can contribute towards the meridional arc. Nevertheless, a very faint arc remains when ray tissue is excluded, and it remains uncertain whether this residual arc is due to overlapping or not. Further pronouncements will be made later in this paper and again in a later work.

In all the species examined, however, the possibility remains that the flattening of the molecular spirals in the walls of tracheids exercises its effect on the meri-

dional arc, not only through the overlapping one has been led to expect in a spiral diagram, but also indirectly through an association of this flattening with some change in the outer wall of the tracheid (to which Bailey & Berkeley attribute this meridional arc), and that this layer does therefore contribute something. In those specimens showing a meridional arc, the outer layers might be thicker than, contain more cellulose than or have its cellulose arranged differently from, those in the specimens in which it is absent. This possibility is examined in the following section. It may be said now that not only is this possibility ruled out, but the results obtained from an examination of the possibility contribute evidence of the most convincing kind that the outer layer in the tracheid wall cannot be wound with chains whose direction is much different from that of the chains in the central layer.

C. Layer thickness and optical properties in transverse sections

For this investigation seven specimens were chosen. The three *Pseudotsuga* samples obviously contributed a series which should be studied. The four other consisted of (a) two specimens (*Pinus insignis* and *Larix leptolepis*) where the diagram shows a rather flat spiral in both but a meridional arc only in one, and (b) two specimens (*Pinus resinosa* and *Picea Abies*) in which the spiral of both is steep but only one shows even a faint meridional arc. If the meridional arc is indeed due to the outer layer, a corresponding variation in the properties of this layer within each of these three groups should be found.

Photomicrographs of four of these specimens in thin transverse section are presented in plates 6 and 7, figures 8, 10, 15 and 17. These were taken under the polarizing microscope between crossed nicols. The thin outer layer will be immediately apparent, and mere inspection even of the limited field presented will show that the variation in the relative thickness is very small. It will further be noticed that the innermost layer, which is also bright, is very much thinner than the outer layer. In view of the small contribution which this layer could give in comparison with the outer layer, this more extensive outer layer only is studied here. Figures for layer thickness determined on numbers of measurements ranging from 30 to 50 are presented in table 1. In every case, the layer thicknesses are derived from a measurement of the total wall thickness of two adjacent tracheids, and the thickness of both outer layers measured together. The latter measurement also includes, therefore, the two primary walls and the middle lamella, but the thickness of these appears so small that no appreciable error is to be anticipated by their inclusion. The latter measurement, divided by two, is taken as representative of the outer layer, and the inner layer is given by the difference between the two measurements divided by two. Hence the number of tracheids measured in each sample ranges from 60 to 100. Determination of the thickness of the outer layer is rendered somewhat uncertain by the higher refractive index of this layer compared with the central layer, with its accompanying Becke line phenomenon. Nevertheless, careful standardization of lighting conditions and focusing technique ensures

that the figures are comparable within themselves, so that a strict comparison can be made within the species.

In table 1 it will be immediately evident that, though there is some variation in the thickness of the outer layers from species to species, this is counterbalanced by changes in the central layer so that the ratio between the two thicknesses remains more or less constant (last column, table 1). In particular, *Pinus insignis* and *Larix leptolepis* are identical in this respect, and so are *Pinus resinosa* and *Picea Abies*. It is clear that, if the meridional arc is derived from the outer layer, its intensity relative to the equatorial arcs depends, in so far as it is related to wall thickness, on the ratio between the layers and not on the values for the layers individually. Here, therefore, is the clearest evidence that the appearance of the meridional arc is not associated with thickness in the visible outer layer of the wall. Similarly, with *Pseudotsuga* there is no clear relationship between the ratio of layer thicknesses and the appearance of the arc in question. Comparing the third and fourth annual ring, for example, the meridional arc is plainly visible in the former where the outer layer is relatively much thinner. Passing to the eleventh ring, a change in the ratio from 3.86 to 4.62 can hardly be sufficient to account for the complete disappearance of the meridional arc.

It remains, therefore, to determine if some feature other than thickness in the outer layer determines the appearance of the arc. Factors such as change in cellulose content, angular dispersion of the cellulose chains within single cells, variations in the distribution of the spiral angle from cell to cell, etc., might perhaps be decisive. The latter property, in particular, could be determined easily by observing the run of striations in longitudinal sections, but in view of the lack of precision in their interpretation this would seem hardly profitable. Actually, a measurement may be obtained which in a way integrates all these factors by determining the double refraction of the outer layers in transverse section. This feature of the wall will be affected by cellulose content, chain direction, angular dispersion, etc., and while variation in double refraction could not easily be associated with changes in any one of these properties, a constant double refraction would indicate that they do not vary widely. The determinations were carried out in light of wave-length 0.59μ ,* the phase difference ϕ was determined by the de Sénarmont compensator with the usual precautions for accuracy and the results converted to double refraction by measuring the thickness d of the section whence double refraction $(n_\gamma - n_\alpha) = \phi(0.59)/360d$. The determinations were made on sections in Canada balsam (and were therefore dehydrated, comparing more closely to the dry specimens used on the X-ray spectrometer than would wet sections), and d was measured by dissolving off the balsam and teasing with fine glass needles until a sufficient number of cells had been turned over on their sides to give a reliable measurement. The results were invariably checked on a second, and sometimes a third, section of different thickness. They are presented in table 2. The

* The light was filtered through Wratten 24A and 52 filters, with a transmission of 1.5 % at 0.59μ and 0.15 % at 0.56 and 0.62μ .

double refraction is always low—much lower than could reasonably be expected from chains approaching the transverse position—and fairly uniform. Comparing, again, the results within the two pairs *Picea-Pinus resinosa* and *Larix-Pinus insignis* and the *Pseudotsuga* group it is seen that in the first pair *Picea* has the lower birefringence and the meridional arc is absent; in the second pair the difference between the figures is hardly significant from the present standpoint; while again in the *Pseudotsuga* group the specimen with the lowest birefringence has the meridional arc completely missing. There is, therefore, at first glance, some sign of a correlation of one of the factors affecting double refraction in the outer layer with the absence of the meridional arc.

TABLE 1. LAYER THICKNESS (μ) IN WALLS OF CONIFER TRACHEIDS AS SEEN BY ORDINARY LIGHT IN THIN TRANSVERSE SECTIONS

species	outer layer thickness (o)		inner layer thickness (i)		ratio (i/o)	
	range	average	range	average	range	average
<i>Picea Abies</i>	0.79-1.30	0.94 \pm 0.04	1.56-5.38	3.46 \pm 0.18	5.60-1.71	3.66 \pm 0.2
<i>Pinus resinosa</i>	0.63-1.43	0.98 \pm 0.05	1.67-5.76	3.26 \pm 0.16	5.60-1.83	3.64 \pm 0.2
<i>Larix leptolepis</i>	0.52-1.14	0.80 \pm 0.03	1.48-3.86	2.54 \pm 0.12	6.74-1.84	3.30 \pm 0.3
<i>Pinus insignis</i>	0.99-1.83	1.32 \pm 0.05	2.32-6.95	4.20 \pm 0.29	6.06-1.38	3.32 \pm 0.2
<i>Pseudotsuga</i> :						
3rd ring	0.35-0.98	0.57 \pm 0.03	1.95-4.27	2.90 \pm 0.10	9.20-3.09	5.33 \pm 0.3
4th ring	0.30-0.98	0.60 \pm 0.03	0.96-4.67	2.14 \pm 0.07	7.90-1.53	3.86 \pm 0.3
11th ring	0.43-1.00	0.61 \pm 0.03	1.48-3.94	2.72 \pm 0.09	8.76-2.23	4.62 \pm 0.2

TABLE 2. DOUBLE REFRACTION IN THE OUTER LAYERS OF CONIFER TRACHEIDS AS SEEN IN TRANSVERSE SECTION IN PARALLEL LIGHT OF WAVE-LENGTH $0.59 \pm 0.03 \mu$

species	double refraction	
	range	average
<i>Picea Abies</i>	0.0020-0.0072	0.0043 \pm 0.0002
<i>Pinus resinosa</i>	0.0029-0.0136	0.0086 \pm 0.0005
<i>Larix leptolepis</i>	0.0078-0.0106	0.0080 \pm 0.0002
<i>Pinus insignis</i>	0.0051-0.0142	0.0097 \pm 0.0004
<i>Pseudotsuga</i> : 3rd ring	0.0106-0.0185	0.0153 \pm 0.0006
4th ring	0.0106-0.0260	0.0153 \pm 0.0005
11th ring	0.0045-0.0105	0.0074 \pm 0.0003

One can, however, now proceed to calculate from these figures the inclination of the cellulose chains to the transverse plane in the outer layer. First, of course, one must know the cellulose content of the layer and the dispersion of the cellulose chains about their mean direction. In conifer wood generally the cellulose content is of the order of 60 % (Allsopp & Misra 1940; Preston & Allsopp 1939; Ritter & Fleck 1926), both for early and for late wood, and since the central layer is well developed only in the latter, this figure would appear reasonable for the outer layers alone. Even assuming that the micelles in the outer layer lie transversely, this

would imply that something like 77 % at least of the thickness in transverse section is cellulose. There is no evidence at the moment concerning the dispersion beyond a general indication that it is not negligible (Preston 1942); but allowance can be made for what would seem to be an inconceivably high value by taking the double refraction of the cellulose as 0.03 instead of the 0.06 which apparently applies to parallel chains (Frey 1926; Kanamaru 1934). It is then a comparatively simple matter to calculate the inclination. The process will be clear from figure 7. Since the index ellipsoid of the cellulose substance is an ellipsoid of rotation, the following relations obtain:

$$(n'_\gamma)^2 \cos^2 \theta / (n_\alpha)^2 - (n'_\alpha)^2 \sin^2 \theta / (n_\alpha)^2 = 1,$$

$$(n'_\alpha)^2 = (n_\alpha)^2.$$

Hence knowing $n'_\gamma - n'_\alpha$, and taking $n_\alpha = 1.53$, θ can be calculated. Since meticulous accuracy here is unnecessary, and in order to avoid laborious calculation, a nomogram

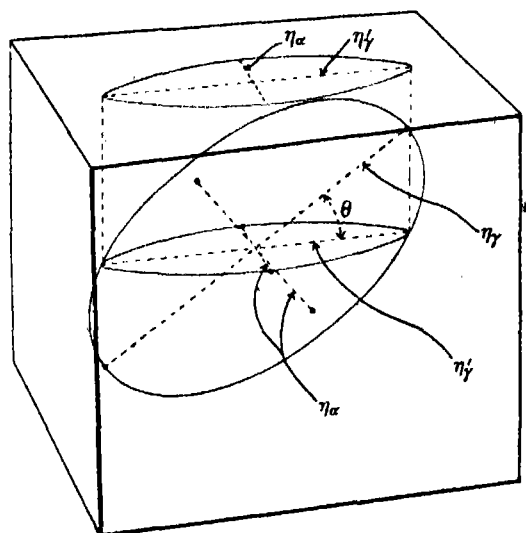


FIGURE 7

was used to derive the figures presented in table 3. For each determination of double refraction four values are given for the inclination, corresponding to an intrinsic double refraction of 0.06 and 0.03, assuming in either case that the cellulose content is either 100 or 77 %. It seems very reasonable to suppose that the true inclination must lie somewhere within the range thus presented and probably lies rather near the figure for 77 % cellulose with a double refraction 0.06. For comparison the inclinations of the chains in the central layer, determined from the X-ray diagrams, are given in the last column of the table. This brings out two striking facts. First, that even making the most favourable assumptions, the cellulose chains in the outer layer are never anything like transverse; and secondly, that the inclination thus calculated agrees closely with that determined from the X-ray diagram for

the central layer. This supports, therefore, the earlier conclusion (Preston 1939 c) on the same point. In other words, the inclination in the outer layer can never be far removed from that in the central layer. The conclusion is therefore inescapable that the outer and inner layers are indistinguishable from this point of view.

TABLE 3. TILT TO THE TRANSVERSE PLANE OF CELLULOSE CHAINS IN THE OUTER LAYER OF CONIFER TRACHEIDS AS DETERMINED FROM OPTICAL PROPERTIES, AND OF THE CENTRAL LAYER AS DETERMINED FROM THE X-RAY DIAGRAM

species	assumed intrinsic double refraction of the cellulose	tilt calculated if the outer layer contains		tilt determined from major 3.9 A arcs
		100 % cellulose	77 % cellulose	
<i>Picea Abies</i>	0.06	75°	74°	73°
	0.03	67°	64°	
<i>P. resinosa</i>	0.06	66°	65°	71°
	0.03	58°	54°	
<i>L. leptolepis</i>	0.06	67°	65°	62°
	0.03	59°	54°	
<i>P. insignis</i>	0.06	65°	62°	58°
	0.03	55°	50°	
<i>Pseudotsuga</i> : 3rd ring	0.06	60°	54°	57°
	0.03	44°	36°	
4th ring	0.06	60°	54°	60°
	0.03	44°	36°	
11th ring	0.06	69°	67°	63°
	0.03	60°	56°	

CONCLUSIONS

There is need, therefore, for little further discussion on the main point at issue. It is now clearer than ever that the optical heterogeneity in the secondary wall of the conifer tracheid is to be sought in some property other than cellulose chain direction. The wall is completely homogeneous in this respect as far as the evidence goes at present; and in particular the presence of a meridional arc in the X-ray diagram of conifer wood is completely spurious. It is obviously imperative that not only must the crystallographic botanist make a careful scrutiny of his material before attempting to interpret his results, but the biologist attempting to use crystallographic methods cannot ignore the basic principles underlying his diagrams without serious danger of gross misrepresentation.

In seeking the cause of the undoubted optical heterogeneity, it must be remembered that this apparently goes hand in hand here with a swelling heterogeneity in the sense that the inner layer swells more readily than the bright outer layer. The writer is not aware of any figures which have been published on this point, and therefore offers the figures in table 4 as an illustration. Preliminary evidence has also been obtained in the writer's laboratory that the central layer in the

tracheid is capable of absorbing much greater quantities of dyes, such as methylene blue, than is the outer layer. These facts recall the interpretation put upon heterogeneity in the collenchyma wall (Majumdar & Preston 1941) as due to changes in *angular dispersion*. As already mentioned in this paper, the writer presented evidence some time ago (Preston 1939c) that the angular dispersion of the central layer is greater than that of the outer layer, and gave a tentative model of the type of change involved in passing from one layer to the other. It would be premature at this time to enlarge on this; but remembering that the disturbance in crystallinity most likely to occur is, without doubt, change in angular dispersion, the following considerations may not be out of place. In the elongated cells of the collenchyma in *Heracleum sphondylium*, which continue to elongate during the deposition of the so-called secondary wall, the outer layer is dark in transverse section and the inner layer bright. The dark outer layer must have been extended considerably, while part at least of the bright layer has had little or no elongation. Together with this goes a slight, but appreciable, discrepancy between the dispersion of the chains in the wall as determined optically from individual cells and in the X-ray spectrometer from bundles, and this has been shown to infer considerable angular dispersion of the cellulose chains somewhere in the wall. In the X-ray diagram, the cellulose chains are shown to run in very steep spirals, and there is not the slightest evidence of any meridional arcs of the type studied in this paper; transverse chains are to all intents and purposes absent. The only possible conclusion appears to be that the chains in the inner layer, laid down after extension has ceased, are more dispersed than those in the outer layer in which the considerable elongation would naturally have reduced any original dispersion which might have occurred. This dispersion would then occur chiefly about a line normal to the wall surface, and the brightness of the inner layer is explained.

TABLE 4. SWELLING OF TRANSVERSE SECTION OF THE SUMMER WOOD FROM *LARIX LEPTOLEPIS* IN SULPHURIC ACID

strength of acid (% wt./vol.)	breadth of cell (scale divisions)	thickness of wall (scale divisions)	increase in dimensions as percentage of original in	
			cell	wall
0	1.107 \pm 0.050	0.141 \pm 0.010	—	—
50	1.266 \pm 0.053	0.225 \pm 0.013	14.2	59.5
60	1.243 \pm 0.070	0.306 \pm 0.015	12.3	117.0
65	1.470 \pm 0.063	0.664 \pm 0.036	32.8	371.0

Each determination is a mean of fifty observations. Notice that the tracheid wall swells enormously with a comparatively slight overall increase in cell diameter, until in 65 % sulphuric acid the lumen is almost fully occluded. This illustrates the presence of an outer layer in the wall whose resistance is limiting overall changes in dimensions which would otherwise be brought about by the swelling of inner layers.

It is certain that few natural cellulosic structures are so perfectly crystalline that no angular dispersion occurs; and since cellulose is invariably laid down in thin cylindrical sheets surrounding a protoplast, the sheets themselves often showing

fine lamellation, the probability is that the dispersion commonly does occur to a greater extent about a line perpendicular to the wall surface than about a line parallel to it. This is the condition assumed to occur in the outer layer in the tracheid wall. Following optical evidence one may then, in the central layer, add to this a considerable dispersion about the line parallel to the wall surface also. This would then make the outer layer bright and the inner layer darker, between crossed nicols, even in tracheids whose micelles are arranged on the average longitudinally, the contrary opinion of Bailey & Berkeley notwithstanding.

Whatever the explanation of optical heterogeneity turns out to be, it is now certain that the bulk at least of the secondary wall is uniform in cellulose-chain direction. This conception, together with the peculiar relation existing between the inclination of this set of chains and the length of the tracheid, led the writer more than ten years ago (1934) to suggest, as a tentative hypothesis, that the walls of the cambial initials from which the tracheids are derived are wound with a somewhat similar molecular spiral. This primary wall would then remain surrounding the secondary layer later deposited upon it, and would add to this secondary layer merely a thinner replica of itself as far as cellulose-chain direction is concerned. Recently, in examining wood for a totally different purpose, the writer has observed a number of very tenuous lamellae standing out from injured tracheids, and these appear to be the primary wall which enveloped the original cambial initial. In these lamellae the cellulose chains run almost transversely. The secondary wall is therefore not a copy of the structure of the primary wall, and the conditions under which the chains of the secondary wall are deposited and oriented demand careful rescrutiny. It is not unsatisfactory that the primary wall here seems to differ from the secondary in this respect, for this brings the tracheid into line with other cell types where the same condition is known to occur. The way is therefore open for a general discussion of the relation between secondary and primary walls; these newer results and their interpretation will, however, be pursued further in the second paper of this series.

The writer is indebted to Miss L. I. Scott, of the Botany Department, for reading through the manuscript of this paper, and to Mr H. J. Woods, of the Textile Physics Laboratory, for the nomogram used in the conversion of double refractions to inclinations. In particular, he wishes to make final acknowledgement of the great debt he owes to the late Professor J. H. Priestley.



FIGURE 8

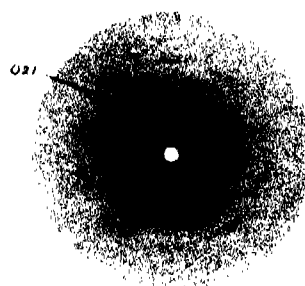


FIGURE 9



FIGURE 10

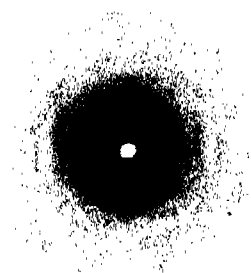


FIGURE 11

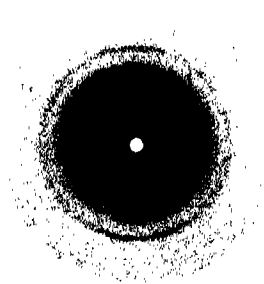


FIGURE 12

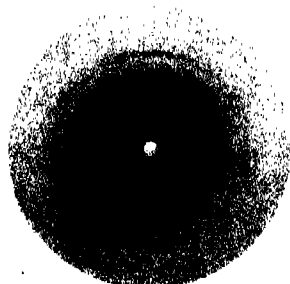


FIGURE 13

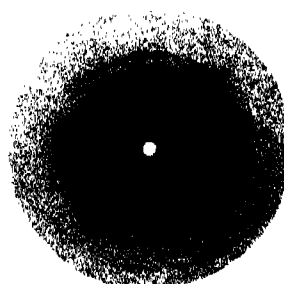


FIGURE 14



FIGURE 15

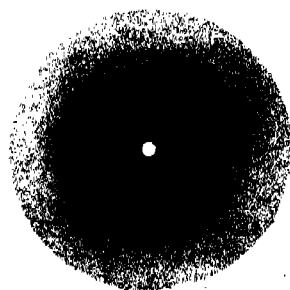


FIGURE 16

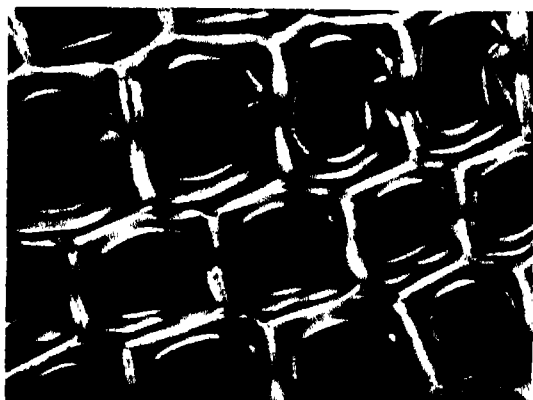


FIGURE 17

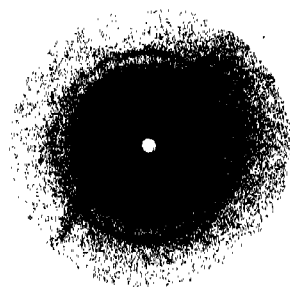


FIGURE 18

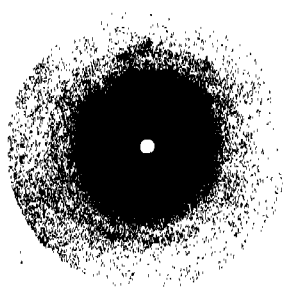


FIGURE 19

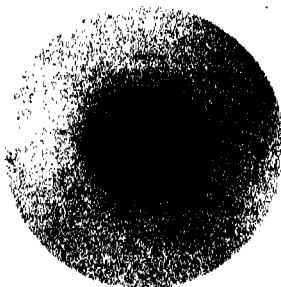


FIGURE 20

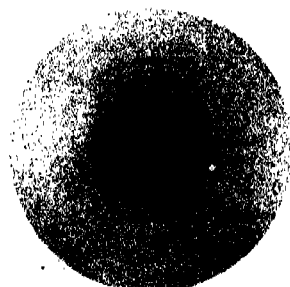


FIGURE 21

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DESCRIPTION OF PLATES

In both plates all X-ray photographs are taken with the beam running radially through the wood and the grain lies parallel to the longer edge of the page. Except where otherwise stated, the piece of wood is 1 mm. thick and the diameter of the beam is 0.5 mm. All photomicrographs are of thin transverse sections between crossed nicols.

Plate 6

- FIGURE 8. *Picea Abies*; section through specimen whose X-ray diagram is given in figure 9.
 FIGURE 9. *Picea Abies*; X-ray diagram. Note complete absence of meridional arcs.
 FIGURE 10. *Pinus resinosa*; section through the specimen whose X-ray diagram is given in figure 11.
 FIGURE 11. *Pinus resinosa*; X-ray diagram. Note the presence of a faint meridional arc of 3.0 Å spacing.
 FIGURES 12, 13 and 14. *Pseudotsuga*; X-ray diagrams of the late wood in the 3rd, 4th and 11th rings respectively. Note the progressive steepening of the spiral in the walls indicated by the decreasing spread of the lateral arcs, and the accompanying steady decrease in the intensity of the meridional arcs.

Plate 7

FIGURE 15. *Pinus insignis*; section through the specimen whose X-ray diagram is given in figure 16.

FIGURE 16. *Pinus insignis*; X-ray diagram. Note the presence of a marked meridional arc.

FIGURE 17. *Larix leptolepis*; section through the specimen whose diagram is given in figure 18.

Note that inner layers are pulled away from the outer layer in some cases.

FIGURE 18. *Larix leptolepis*; X-ray diagram. Note the absence of a meridional arc.

FIGURE 19. *Juniperus virginiana*; X-ray diagram. Note the wide spread of the lateral arcs and the appearance of a marked meridional arc.

FIGURES 20 and 21. *Pinus resinosa*; X-ray diagrams of a section 300μ thick with a collimating slit 0.1 mm. diameter. In (20) the X-ray beam passes through a region free of ray tissue; in (21) a ray is included in the specimen photographed. Note the stronger appearance of the meridional arc in the latter diagram.

Note that the following X-ray diagrams form a sequence in which the spread of the lateral arcs steadily decreases accompanied by a steady diminution of the intensity in the meridional arcs: plate 7, figures 19, 16, 18; plate 6, figures 11 and 9.

A study of an insect cuticle: the larval cuticle of *Sarcophaga falculata* Pand. (Diptera)

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(Communicated by H. Graham Cannon, F.R.S.—Received 25 April 1945)

[Plate 8]

The rate of growth of the larval cuticle of *Sarcophaga* and the chemical composition of its layers are fully described.

The cuticle consists essentially of two fundamentally different layers. The outer layer—the epicuticle—is about 4μ thick, and contains no chitin. It is basically composed of protein, having its isoelectric point at pH 5.1. At its surface a resistant lipo-protein complex forms a very thin membrane which may be separated intact from the remainder of the epicuticle which contains little or no lipid. The epicuticle is therefore a double structure.

The inner layer of the cuticle—the endocuticle—is much thicker than the epicuticle, and is a laminated chitin-protein complex of isoelectric point pH 3.5. Like the epicuticle it is a double structure. The outer zone of the endocuticle is secreted during early larval life, and is penetrated by pore canals which do not enter the epicuticle. The inner zone of the endocuticle is secreted later in larval life, and contains no pore canals. An exocuticle is absent from the larval cuticle, but the outer endocuticle is destined to form the exocuticle of the puparium, the inner endocuticle remaining unchanged.

The pore canals at first contain cytoplasmic filaments, but these later become coiled and replaced by chitin.

I. INTRODUCTION

During the past fifteen years the claim made over a century ago (Odier 1823) that the insect cuticle contains protein and other substances in addition to chitin has been repeatedly confirmed (Kühnelt 1928*a, b*; Campbell 1929; Evans 1932, 1934, 1938;

Wigglesworth 1933; Trim 1941), and it has become abundantly clear that the cuticle is not a simple investment of chitin, but is of considerable chemical complexity. Pryor (1940*a, b*) has shown that the conversion of a soft white cuticle into the hard and dark armour of the puparium or mature insect is not the result of impregnation with 'incrusting substances' as had been previously suggested (Schulze 1922; Kühnelt 1928*a*), but is largely due to the 'tanning' of its proteins by the coloured oxidation products of a phenol secreted into the cuticle at the time of hardening. Moreover, Fraenkel & Rudall (1940) have shown that concurrently with this process pronounced orientation of chitin crystallites takes place within the protein phase of the cuticle, and this, together with loss of water and loss of solubility of the protein, results in the formation of a hard and dense structure. The changes involved in hardening affect only the outer zone of the cuticle, and result in the formation of a brown or black exocuticle, the inner zone persisting unchanged as a soft white endocuticle.

In addition to these two important constituent layers of the fully differentiated cuticle a thin resistant epicuticle overlies the exocuticle. It contains no chitin, and owes its protective qualities not only to the presence of fatty acids and cholesterol (Kühnelt 1928*a*), but also of paraffins of the order C_{27} - C_{31} (Bergmann 1938).

In spite of much fundamental work, however, many outstanding questions remain to be answered, since histological examination has not kept pace with advances made in the study of the chemistry of the cuticle. These advances have often been the result of work on whole cuticles or on extracts, and information on the location of the substances identified has therefore been lacking. The precise relation between the well-known facts that the enzyme tyrosinase is abundant in insect blood and other tissues, and that darkening of the cuticle involves enzyme activity, has not been demonstrated, although (following Dewitz 1902, 1905, 1916) it has been generally assumed that the relation is an intimate one. Furthermore, it has not been shown why darkening of the cuticle proceeds inwards from the epicuticle, although the oxygen consumed in the process is supplied not from the atmosphere directly, but from within the body by way of the tracheal system (Fraenkel 1935), and clearly the chromogen involved must also be supplied from within the body. The onset of pupation in Diptera, accompanied by the hardening and darkening of the cuticle of the third larval instar to form the puparium, has been shown to be due to the liberation of a hormone (Fraenkel 1935; Becker & Plagge 1939), the mode of action of which, however, is obscure. The fine pore canals which rise vertically through the cuticle (Leydig 1864; Holmgren 1902; Plotnikow 1904; Berlese 1909; Wigglesworth 1933) deserve further study, particularly with regard to the nature of their contents. And finally the importance of the epicuticle in controlling water loss through the cuticle and the permeability of the cuticle to oil-borne insecticides (Hurst 1943*a, b*; Alexander, Kitchener & Briscoe 1944*a, b*; Wigglesworth 1944*a, b*) points to the need for further examination of this layer.

In an attempt to give at least a partial answer to these questions a detailed study has been made of the cuticle of the third instar larva, and of the puparium, of *Sarcophaga falculata*. The reasons for selecting this insect for study were that it is easily bred and provides a generous supply of larvae, the cuticle is thick yet readily handled and sectioned, and it provides admirable material for studying darkening and hardening of the cuticle. In addition it has, with *Calliphora erythrocephala*, already received considerable attention. Pryor (1940b) studied the formation of the puparium of *Calliphora*, and Fraenkel & Rudall (1940) have given an account of the physical and chemical properties of the larval cuticle of *Sarcophaga*. Trim (1941) has characterized the proteins obtained from larval cuticles of *Sarcophaga*. Earlier, Fraenkel (1935) had described experiments on *Calliphora* which showed a connexion between the liberation of a hormone and the formation of the puparium. And finally, dipterous larvae have already been the subject of work on tyrosinase and melanosis of the cuticle (Dewitz 1902, 1905, 1916; Gessard 1904; Graubard 1933). It was therefore hoped that a histological and histochemical study of the larval cuticle of *Sarcophaga* would lead to considerable integration of the information presented by these authors.

The present paper is devoted to an account of the larval cuticle, and in a later paper a detailed account will be given of the changes taking place during the formation of the puparium.

A preliminary note on the pore canals has already been published in *Nature* (Dennell 1943), and the events taking place at pupation have been outlined in a later account in the same journal (Dennell 1944).

II. METHODS

It was essential throughout the work to have an abundant supply of larvae of known age reared under reproducible conditions. Flies were therefore kept in an illuminated cage maintained at 28° C and 65 % R.H. in a large incubator, and on a diet of cane-sugar and water lived healthily. Addition of protein in suitable form is necessary, however, for mating and the deposition of larvae, and was originally supplied as horse-flesh. Later a proprietary dog food proved satisfactory, but ultimately moistened fish-meal, on which larvae are deposited freely and grow well, was used exclusively on account of its great convenience. The following procedure for obtaining larvae was adopted. Fish-meal was supplied to the flies until unsegmented eggs were shed, an indication that the production of active larvae was imminent. The fish-meal was then removed, and after an interval of 1 or 2 days its re-presentation for a short period usually resulted in the deposition of a generous supply of larvae. The larvae were reared at 28° C in batches of fifty in 1 lb. jars containing 50 g. of fish-meal moistened with 50 c.c. of water, and ultimately allowed to pupate in dry sawdust. Their rate of growth under these conditions will be described in the following section.

For histological work on the cuticle paraffin, frozen and hand-sections were used, and comparison of these formed a valuable check on the accuracy of observations. Dioxan (diethylene dioxide) over calcium oxide was used for dehydration in the preparation of paraffin sections, the hardening of the cuticle caused by the use of absolute alcohol being thus avoided. For sections cut by the freezing method pieces of cuticle were infiltrated successively with 12.5 and 25 % gelatine solution at 37° C, and later embedded in 25 % gelatine hardened in 10 % formalin. Blocks of good consistency were obtained. They were frozen thoroughly after rinsing in distilled water, and sectioned while thawing. Sections of 15 μ were obtained, and after staining, usually with Sudan Black B (see Lison 1936) for fatty substances, were mounted in 'Glychrogel' or Apathy gum solution. Hand-sections of the fresh cuticle were obtained by rolling a sheet of cuticle and cutting the roll transversely while held against the thumb. These sections proved invaluable for work on the polyphenol oxidase of the cuticle and for experiments with various substrates.

Of many fixatives tried, Flemming without acetic, Gilson's mercurio-nitric mixture and Carnoy-Lebrun gave the best results. For the cytoplasm of the pore canals of the young larva Flemming was unequalled, and penetration of the fixative was greatly improved by the addition of 0.9 % NaCl.

Mallory's triple stain proved admirable for the paraffin sections. Delafield's haematoxylin and picro-indigo-carmin were also used, but to a less extent. The value of Mallory's stain consisted in its perfect and constant differentiation of the epicuticle and in the clear distinction obtained between the cytoplasmic and cuticular contents of the pore canals. Other stains employed for comparison will be mentioned in passing.

Considerable use has been made of histochemical tests. In the hands of previous workers they have given valuable information on the nature of the constituents of the cuticle, but here, owing to the ease with which hand-sections of fresh larval cuticles may be obtained, it has been possible to extend their use to a study of the polyphenol oxidase of the epicuticle. The chemical tests employed will be mentioned appropriately in the text.

III. THE GROWTH OF THE LARVA

No significant differences in size have been observed between the many broods of larvae which have been reared during the course of this work. Under the conditions noted in the previous section the first and second larval instars are of only short duration, and the third and last instar begins at about 36 hr. after the larvae have been deposited. The three instars are easily distinguished, apart from the difference in size, by the form of the posterior spiracular openings. In the first instar larva each tracheal trunk opens by a simple pair of stigmatic slits, in the second by a similar but larger pair almost surrounded by a crescentic peritreme, and in the third by three slits surrounded by the peritreme. Just before the moult between second and third instars the characteristic tracheal armature of the third

instar is seen underlying that of the second, and a convenient datum point for assessing the beginning of the third instar is therefore available.

The third instar larva grows rapidly (figure 3) for about 2 days, during which the crop becomes greatly distended and shows through the transparent cuticle. When the larva has attained its full size the crop is progressively emptied, and after about a further $1\frac{1}{2}$ days is no longer visible. The mature larva passes through a 'resting period' of about 1 day before pupation begins. The durations of the larval stages differ somewhat from those noted by Hafez (1940).

During larval growth there are therefore well-defined stages which make the assessment of age an easy matter. It will often be more convenient to refer to 'larva, crop full', 'larva, crop empty', and so on, than to quote the actual age of the larva. Where the term 'larva' is used without qualification the third instar larva is implied.

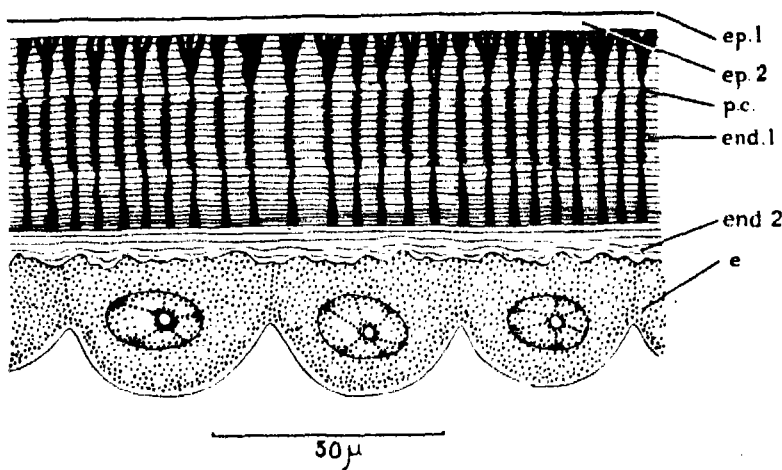


FIGURE 1. The larval cuticle at a little over 2 days, based on information from Mallory and chitosan preparations. e, epidermis; end. 1, outer endocuticle; end. 2, inner endocuticle; ep. 1, outer epicuticle; ep. 2, inner epicuticle; p.c., pore canal.

IV. THE DEVELOPMENT AND STRUCTURE OF THE CUTICLE

The cuticle of the third instar larva of *Sarcophaga* consists of two primary layers, the epicuticle and the endocuticle. The epicuticle is secreted first in the development of the cuticle, before the cuticle of the second instar is shed, and soon attains its full thickness of about 4μ . The endocuticle, on the other hand, continues to be laid down throughout larval life, and even for a short time after pupation. There are two distinct phases in the secretion of the endocuticle, and distinct inner and outer zones of this layer are to be recognized. The first phase begins somewhat before the epicuticle has reached its full thickness and continues until a layer of endocuticle about 40μ thick has been produced (figure 1). This layer contains pore canals and, as will be seen later, is distinguishable in many ways from the inner layer, devoid of pore canals, which now begins to be added.

Paraffin sections of larvae which bear the spiracular armature of the second instar overlying that of the third, stained in Mallory's triple stain, show the epicuticle as a deeply staining red layer, whereas the underlying endocuticle stains more lightly with aniline blue. The cuticle of the second instar overlies but is separated from that of the third, and consists of intact epicuticle and the almost completely digested remains of the endocuticle. The early growth of the third instar cuticle and the shedding of that of the second instar appear to take place in a manner similar to that described by Wigglesworth (1933) for *Rhodnius*. The new epicuticle appears to be secreted by the epidermis and not by special tegumental glands as in the 'cuticle' of *Homarus* (Yonge, 1932). No dermal glands for the secretion of a moulting fluid have been observed at this stage, but if they behave as in *Rhodnius* it is possible that by this time they have already degenerated. By the time the old cuticle is shed, the new cuticle of the third instar is about 10μ thick, and during the next $1\frac{1}{2}$ days it increases steadily in thickness to about 40μ . It shows distinct horizontal striae suggesting a laminated structure.

While examining paraffin sections of third instar larvae about 2 days after deposition the pore canals were first noticed. They appear as red-staining irregular filaments, apparently cytoplasmic, projecting from the epidermis and rising some way into the endocuticle. Close examination showed that each filament is continued through the remainder of the endocuticle as far as the epicuticle as a delicate rod, which stains lightly with aniline blue and is therefore almost indistinguishable from the surrounding endocuticle (plate 8, figure 6). Tangential sections give further information. Both acidophil and basophil portions of the filaments appear as discrete structures occupying but not, apparently, entirely filling tubular canals in the endocuticle, so that a small annular space occurs between the wall of the canal and its contents. The appearance of the distal portions of the filaments suggests that they are composed of cuticular material. Numerous authors (for example, Leydig 1864; Tower 1906; Hass 1916; Poisson 1924; Wigglesworth 1933) have described cytoplasmic filaments extending from the epidermis into the cuticle in a number of insects, and apparently remaining in this condition throughout life, while Holmgren (1902), Plotnikow (1904), Berlese (1909) and Eder (1940) have expressed the view that these filaments are ultimately transformed into cuticular substance. A detailed study of the canals and their contents in *Sarcophaga* was therefore called for. It is important to notice, as Wigglesworth (1933) has pointed out, that the evidence adduced for the cuticularization of the pore canal contents is slender in the extreme, consisting merely of the similarity of the pink staining of the cuticle and the contents of the canals when haematoxylin and eosin have been used. But, on the other hand, the filling of the pore canals with air when a section is allowed to dry is itself only partially satisfactory evidence that the canals contain protoplasm. Wigglesworth (1933) makes no claim that it indicates more than that the canals are filled with protoplasm or with some fluid, but Richards & Anderson (1942) use similar evidence to support their contention that the canals are filled, not with protoplasm, but with a fluid, probably a salt solution,

in equilibrium with the underlying epidermal cells. As will be seen from the following account the statement that the canals are filled with cuticular substance is correct for *Sarcophaga* larvae at least.

In studying the pore canals portions of larvae were fixed at regular intervals in Flemming without acetic from the beginning of the third instar until pupation, and after sectioning were stained in Mallory's triple stain. At the same times the cuticles of other larvae of the same brood were heated to 160° C for 30 min. in potash solution saturated at room temperature. They were then washed, embedded in paraffin, and after sectioning were treated with 0.2 % iodine solution followed by 1 % sulphuric acid. This modification of the original van Wisselingh procedure gives a specific test for the presence of chitin, based on the conversion of this substance to chitosan (Campbell 1929). Its application here clearly revealed the presence of chitin in the pore canals of older larvae. Later, for demonstrating the presence and form of the canals rather than the composition of their contents, the procedure recommended by Schulze (1922, 1924) was found valuable and easier to apply than the chitosan test. In this procedure cuticles were first treated with diaphanol (chlorine dioxide in glacial acetic acid), sectioned, and then treated with a solution of zinc chloriodide. It is claimed that the violet colour given by chitin constitutes a specific reaction, but Kühnelt (1928*b*) denies this, and Campbell (1929) in his critical survey of the methods employed for the detection and estimation of chitin states that results of the test should be interpreted with caution. In the present experiments a chestnut brown instead of violet coloration was usually given by the contents of the pore canals of *Sarcophaga* larvae. The value of Schulze's test, however, lies in the fact that the epicuticle and epidermis remain intact, whereas in the Campbell method they are destroyed.

The information given by the Mallory and the chitin preparations is complementary and unambiguous. The Mallory-stained sections show the contents of the pore canals of the youngest larvae as delicate strands rising from the epidermal cells and passing into the endocuticle. Each strand soon breaks up into a tuft of fine filaments extending to but not entering the epicuticle (plate 8, figure 5). The filaments stain prominently with acid fuchsin, and closely resemble those illustrated by Plotnikow (1904) in the larva of the silkworm and of a Syrphid. In larvae a little older single cytoplasmic strands, apparently coiled, leave the epidermal cells and pass into the endocuticle, but fail to reach its outer border (plate 8, figure 6). In larvae yet older the strands are still shorter, and finally, in larvae about 3 days after deposition, when the cuticle is about 40 μ thick, they are no longer visible.

The potash-treated sections, tested for the presence of chitosan, present further information. In the youngest larvae examined, which in Mallory-stained sections show the red-staining strands extending completely to the epicuticle, the endocuticle gave a rather pale but definitely positive reaction, but no indication of the pore canals was apparent. In older larvae only the outer ends of the canals gave a positive reaction, stronger than that of the endocuticle, and finally, in larvae

about 3 days old, the whole extent of the canals was revealed by a very deep violet coloration. In all larvae of the same age up to 3 days the sum of the extents of the canals as revealed by the two methods was equivalent to the thickness of the endocuticle. In larvae of about 3 days, however, it was often found that the chitinized pore canals did not extend fully to the inner border of the endocuticle (plate 8, figure 7). This was due not to the fact that withdrawal of the cytoplasmic filaments of the pore canals was incomplete, but that after their withdrawal the inner zone of endocuticle, lacking pore canals, began to be secreted. The growth of the inner endocuticle will be discussed later. The appearance of the cuticle when the contents of the canals are completely replaced by chitin and endocuticle growth has continued for a short time is seen in figure 1.

An indication that the red-staining contents of the canals seen in the Flemming-fixed sections are truly cytoplasm is given by an examination of cuticles which had been treated with 10% potash solution at 100° C for an hour before being sectioned and stained in Mallory. In all but the youngest larvae the distal ends of the canals are filled with chitinous plugs staining with aniline blue like the endocuticle itself. The basal ends, however, are completely empty, as would be expected if they had contained cytoplasm. More satisfactory evidence is given by boiling hand-sections of fresh cuticles in a 0.2% solution of ninhydrin (triketohydrindene hydrate) which in the presence of α -amino acids gives a deep blue coloration (Lison 1936). Over small extents of the cuticle the basal contents of the pore canals become prominently coloured by this treatment.

In general, the staining reaction of the cytoplasm of the canals with a variety of dyes is similar to that of the epicuticle, which, as will be seen later, is basically composed of protein. The significance of this similarity will be considered in discussing the possible functions of the pore canals.

Whether the cytoplasmic filaments of the canals are actually progressively withdrawn, secreting chitin from their tips and so leaving behind them strands of this substance, or whether they themselves become progressively chitinized from their distal ends inwards, is not clear. But the result of either process is the formation of chitinized plugs accurately moulded to the form of the canals, and, in chitosan preparations, revealing it with perfect clarity (figure 1 and plate 8, figure 7). Approximately the outermost third of each canal is relatively broad, and often splits into a number of branches. The central third is slender and closely coiled, often so closely that it resembles a more massive but uncoiled strand. The innermost third of the canal is again broader and uncoiled, but is always undivided.

The resemblance of these canals to those of the cockroach *Periplaneta americana*, described by Richards & Anderson (1942) as the result of a study with the electron microscope, is at once apparent. The pore canals of the cockroach also are coiled, but show no distal expansion or splitting. It is claimed that they are filled, not with cuticular substance, but with some fluid. In size the canals of the two insects are markedly different, those of the cockroach having an average diameter of 0.15μ , and being coiled into a helix of pitch 0.25μ , whereas those of *Sarcophaga*

have a diameter of about 1.0μ and a pitch of about 2.5μ . This difference in size cannot be explained by a difference in the thickness of the cuticles of the two insects, for in both it is about 40μ thick.

Further indication of the solidity of the pore canal contents in *Sarcophaga* is provided by splitting horizontally the fresh cuticle of a larva about 3 days old. The pore canals of the two sheets so obtained are readily seen in surface view. Usually the outer sheet bears on its inner face the projecting stiff contents of the canals, while the inner sheet shows corresponding empty canals. The ease with which the contents are withdrawn from the canals suggests that there is no fusion between them and the surrounding endocuticle, and confirms the observation previously made that an annular space may exist between wall and contents of the canals. These fresh preparations give immediate information on the number of canals associated with each epidermal cell, for if the cuticle is handled carefully and examined in Ringer solution the cells remain in contact with the cuticle and their outlines are readily seen. The canals are arranged in clearly distinct fields corresponding to the distribution of the underlying cells, and as many as 50–70 canals are associated with each cell. Beneath the low rounded spines of the cuticle the canals are more closely crowded than in adjacent areas. The density of distribution of the canals is of the order of at least 15,000 per sq.mm., a low figure in contrast with that of 1,200,000 for the much finer canals of the cockroach.

The foregoing observations represent, apparently, the first unequivocal demonstration of the nature of the contents of the fully formed pore canals of any insect. The universal cuticularization of pore canals is, however, not necessarily implied, for the larval cuticle of *Sarcophaga*, being destined ultimately to be detached from the epidermis but retained as the puparium, may well show early specialization to this end. It seems reasonable to suppose that although during the early development of an insect cuticle the canals are always filled with cytoplasm, the later history of these structures may show considerable divergences in different insects.

With regard to the function of the pore canals it is generally agreed that, whatever their ultimate fate, they are concerned with the secretion of the cuticle. Wigglesworth (1933), referring to the endocuticle of *Rhodnius*, states: 'The exact mechanism of its formation is not known, but it must evidently be separated from the cells in fluid form around the protoplasmic filaments that occupy the "pore canals", because there is no sign of any partitioning ("Felderung") of the endocuticle to correspond with the underlying epithelium, such as occurs in those cases where the cuticle arises by a transformation of the superficial layers of the epidermal cells.' But it must be remembered that endocuticular material may be secreted in the absence of pore canals; in the later development of the larval cuticle of *Sarcophaga* the inner endocuticle continues to grow after the pore canals are fully formed, chitinized, and detached from the epidermis. Even here there is no sign of the partitioning mentioned by Wigglesworth. Clearly the endocuticle is secreted in fluid form from the surface of the epidermis, and does not arise by transformation of superficial layers of these cells. In this connexion it is interesting to note that

Eder (1940) and Richards & Anderson (1942) report the absence of pore canals in soft larvae, and the latter authors, referring to the result of an investigation by Alexandrov (1935), state that 'Presumably the species he used have no pore canals (fly larvae, probably similar to mosquito larvae, see below),...'. Lowne (1890-2), however, clearly refers to fine processes which pass from the epidermal cells into canals in the endostracum (endocuticle) of the larva of *Calliphora erythrocephala*. He furthermore describes the endocuticle as being divided by vertical planes into hexagonal prisms, but unfortunately in his illustrations the planes of separation seem to correspond with the pore canals. In the present work no cuticular prisms have been seen in either *Calliphora* or *Sarcophaga*, and *Calliphora* has pore canals like those of *Sarcophaga*.

Since endocuticle secretion may proceed in the absence of pore canals the suspicion arises that the canals may have functions unconnected with the production of this layer. It has been observed in *Sarcophaga* that chitinization of the distal contents of the pore canals does not begin until the epicuticle has attained its full thickness, and it seems possible that one function of the canals may be to assist in the production of this layer. As will be shown later the epicuticle is actually a double structure, consisting of an outer very thin resistant layer (figure 1, *ep.* 1), possibly corresponding to the epicuticle of other authors, and a much thicker inner layer consisting mainly of protein (figure 1, *ep.* 2). Richards & Anderson (1942) describe a similar double epicuticle in the cockroach. It is possible that the outer layer of the epicuticle in *Sarcophaga* is secreted by the surface of the epidermis, and that the inner layer is formed wholly or in part by secretion from the tips of the cytoplasmic filaments of the canals. Some support, admittedly slight, for this suggestion is given by the similarity often seen between the staining reactions of the inner epicuticle and the cytoplasmic filaments. In Carnoy-fixed sections stained with micro-indigo-carmin both the inner epicuticle and the filaments stain a distinct blue-green, but the cytoplasm of the epidermal cells is red. Similarly, in formol-fixed sections stained with Mallory the epicuticle and filaments are a clear red, but the epidermis is purple.

Other functions which have been ascribed to the pore canals are the transport of an oxidizing enzyme⁴ to the epicuticle (Wigglesworth 1938, 1939), and the transport soon after ecdysis of a protein and a phenol which react in the outer part of the cuticle to form the amber-coloured material of the exocuticle (Pryor 1940b). These suggestions will be examined in a later paper in describing the formation of the puparium of *Sarcophaga*.

The growth of the cuticle after the complete chitinization of the pore-canal contents is interesting. Not only does secretion of the inner layer of endocuticle begin when the canals are fully chitinized, but the outer layer of the endocuticle begins very rapidly to increase in thickness. The appearance of the cuticle at about 3 days has been shown in figure 1. The outer endocuticle then is about 40μ thick, and the inner endocuticle has already reached nearly 10μ . During the succeeding day the outer endocuticle increases to no less than about 140μ , while the inner

endocuticle has continued its growth and now forms a conspicuous layer over 40μ thick. After the fourth day, however, no further growth of the outer endocuticle takes place, but the inner layer continues to grow, and immediately before pupation has reached a thickness of nearly 80μ , approximately a third of the total thickness of the cuticle (figure 2). It continues to grow for some time after the puparium has begun to form. These features of the growth of the cuticle are illustrated in figure 3.

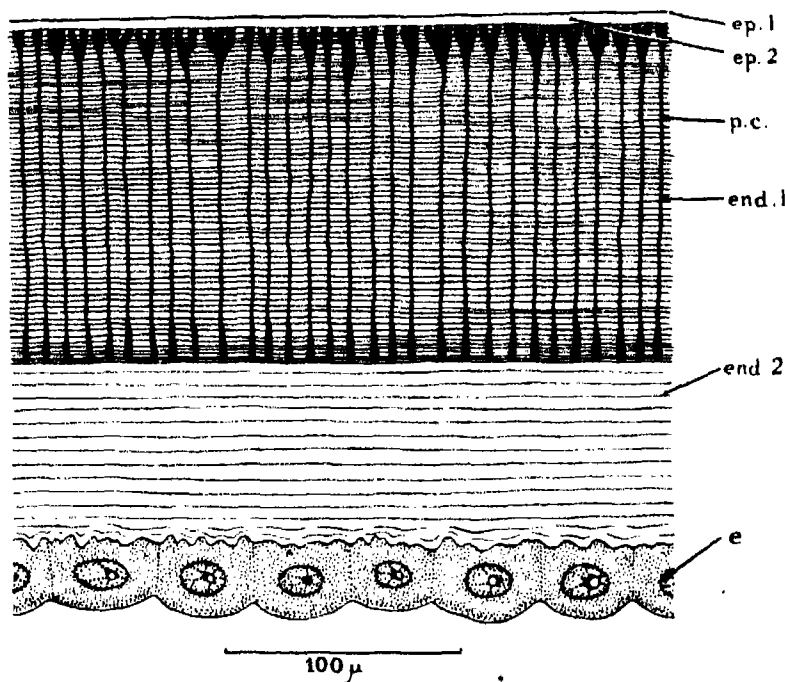


FIGURE 2. The cuticle of the mature larva. Mallory and chitosan preparations. Abbreviations as in figure 1.

Profound differences between the two layers of endocuticle will be detailed in giving an account of the chemistry of the cuticle and in describing the formation of the puparium, but at present it is sufficient to state that while the outer endocuticle stains readily with aqueous methylene blue the inner stains only lightly or not at all; and that in addition to being devoid of pore canals the inner layer shows a rather coarser lamination than the outer.

Wigglesworth (1933) has described two clearly defined layers of endocuticle in the fourth nymph of *Rhodnius*, in which the outer half is laid down completely before moulting, and the inner half after the moult. The pore canals, however, penetrate both layers. Kühn & Piepho (1938) state that secretion of the endocuticle takes place at the same time as hardening and darkening of the cuticle in *Ephestia kuehniella*, a condition comparable with the continuation of growth of the endocuticle after the onset of puparium formation in *Sarcophaga*.

The growth of the outer endocuticle of the larva of *Sarcophaga* after chitinization of the pore canals is complete is clearly reflected in the change of form of these structures. At the third day the canals are helicoidal (figures 1, 3), but at the fourth day, when the outer endocuticle has increased in thickness more than threefold, they are drawn out and become almost straight (figures 2, 3). It is clear that an already existing layer of endocuticle expands, and is not increased in thickness merely by increment at its inner surface. The separation of the outer endocuticle from the epidermis by the intervention of the growing endocuticle renders this latter mode of growth improbable.

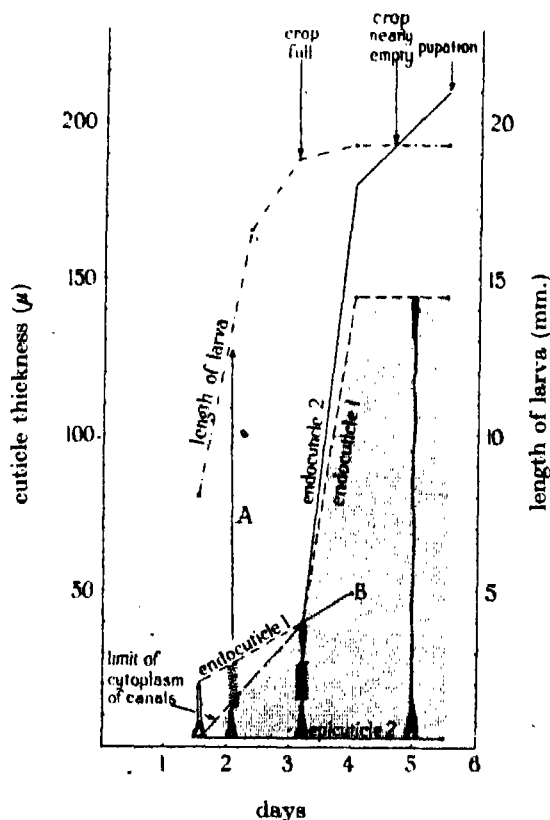


FIGURE 3. The rate of growth in thickness of the layers of the larval cuticle, and the growth in length of the larva. The cuticle is represented as inverted, the epicuticle lying along the abscissa. The extent of the chitinized pore canals is indicated by shading, and the extent of the cytoplasmic filaments by the unshaded area of the outer endocuticle (1).

Before leaving the morphology of the cuticle attention may be drawn to the figure of the larval cuticle of *Calliphora erythrocephala* given by Frænkel & Rudall (1940) in which the epicuticle is indicated as the exocuticle. The formation of an exocuticle, however, is conditional on the process of hardening and darkening of the cuticle, which does not take place until the formation of the puparium. The

term exocuticle, denoting a brittle pigmented outer layer, was suggested by Mr R. E. Snodgrass (Campbell 1929), and Wigglesworth (1933) clearly defines the exocuticle as an outer pigmented layer of the endocuticle, present only in rigid portions, and containing chitin. As will be seen later, the layer identified as the exocuticle by Fraenkel & Rudall contains no chitin. Pryor (1940*b*) also mentions the exocuticle of the same larva, but it does not appear that he is confusing this layer with the epicuticle, as suggested by Richards & Anderson (1942), for he clearly refers to the fact that the epicuticle gives a positive argentaffin reaction whereas the exocuticle does not. His exocuticle may be the outer layer of the endocuticle. In his illustration (plate 17, figure 4) the layer giving the argentaffin reaction can only be the thick epicuticle.

V. THE RATE AND MODE OF GROWTH OF THE CUTICLE

In order to discover whether the rapid increase in thickness of the cuticle between the third and fourth days is due to an increase in the rate of cuticle secretion, or to other causes, a study has been made of the rate of increase in both thickness and weight of the cuticle. Measurements of cuticle thickness have been made at intervals on unstained hand-sections of fresh cuticles of a number of larvae. Errors due to contraction of the cuticle on fixation and preparation for sectioning have therefore been avoided, but it is possible that the hand-sections swell somewhat on mounting in water. The pore canals are readily seen in hand-sections as refringent rods in all but the youngest larvae, and the two layers of the endocuticle are therefore distinguished with ease. Measurements of cuticle thickness have been extended to the puparium, but consideration of their significance will be deferred to a later paper.

The results of measurements of cuticle thickness are shown in figure 3. The cuticle increases steadily in thickness during the first $1\frac{1}{2}$ days of the third instar, and during this period the cytoplasm of the pore canals is progressively replaced by chitin. At about the third day, when the canals are fully chitinized, secretion of the inner endocuticle begins, and at the same time the outer endocuticle begins to increase rapidly in thickness. As a result the total thickness of the cuticle increases enormously between the third and fourth days of larval life. By the fourth day the outer endocuticle has reached its full thickness of about 140μ , but the inner endocuticle continues to grow and reaches a thickness of about 70μ immediately before the formation of the puparium. In figure 3 the point *B* indicates the thickness which would have been attained after 4 days if the cuticle had continued to thicken at its former rate: actually by this time it reaches a thickness of no less than about 180μ .

Observations have therefore been made on the increase in weight of the cuticle during larval life, and have been conveniently combined with quantitative estimates of the major constituents of the cuticle. The methods used have been those which Fraenkel & Rudall (1940) applied to a comparison of larval with pupal cuticles,

with the important exception that a different method of preparing the cuticles in the first place has been employed. Fraenkel & Rudall report considerable amounts of water-soluble material, chiefly protein, in the cuticle, and it was felt that their method of preparation, involving heating the larvae in water to 65° C and scraping the cuticles clean under water, might not only remove some of these materials but perhaps result in a changed water content. Larvae were therefore opened without being previously killed and the cuticles scraped clean under medicinal liquid paraffin. The paraffin did not obviously penetrate the cuticles and appeared to be completely removed when they were firmly pressed between filter papers before being weighed and dried. It is noteworthy that cuticles prepared in this way show a lower wet weight and therefore a lower water content than do cuticles cleaned under water. The dry weights of cuticles prepared by the two methods show no significant difference if the water-cleaned cuticles are immersed for only a short period, and it therefore appears that no paraffin is absorbed on the one hand, but that water is absorbed on the other hand.

Cuticles prepared under liquid paraffin consistently show a water content close to 55 %, whereas Fraenkel & Rudall report a water content of about 70 %. It is clear that in the estimation of the water content the results obtained vary with the methods adopted for the preparation of the cuticles. The chitin content of paraffin-prepared cuticles approximates to 20 % of the wet weight, or about 42 % of the dry weight. Fraenkel & Rudall (1940) originally recorded a chitin content of 60 % of the dry weight in cuticles of *Sarcophaga* larvae, but now, in a private communication, they state that recent work with improved methods shows that chitin amounts to 52 % of the dry weight (equivalent to 15.6 % of the wet weight). Chitin contents of other cuticles have been recorded by Campbell (1929) for the cockroach *Periplaneta americana* (22-60 % in different regions of the body), Kuwana (1933) for the silkworm (10-20 % in exuviae), and Tauber (1934) for the cockroach *P. fuliginosa* (18-37 %).

The primary object of the present study, however, is not to obtain information on the constituents of the cuticle but to throw light on the mode of increase in its weight during the period occupied by its rapid increase in thickness. From the foregoing it is evident that the quantitative study of cuticular constituents presents difficulties, and it is claimed only that the methods adopted in the present work give reproducible results and indicate the trend of cuticle growth during the period under review. With this in mind, a detailed quantitative comparison of the results of thickness and weight measurements will not be undertaken.

Figure 4a illustrates typically the mode of increase in weight of the cuticle of *Sarcophaga* during larval growth. Both wet and dry weights rise smoothly with some approach to a logarithmic mode of increase, and no sharp deviation from this mode capable of explaining the sudden expansion of the cuticle is apparent in the period 3-4 days. There is clearly no significant change in the water content, nor in the content of chitin and protein, in this period, and the possibility that the cuticle suddenly expands as a result of absorption of water is therefore excluded.

It necessarily appears that new cuticular substances in constant proportion must be added not only to the inner endocuticle, but to the outer endocuticle also. Owing to the separation of the outer endocuticle from the epidermis by the growing inner endocuticle these substances must pass through the inner endocuticle in soluble form. It is interesting to note that Trim (1941) has suggested that chitin may crystallize from a homogeneous fluid polysaccharide-protein complex secreted by the hypodermis.

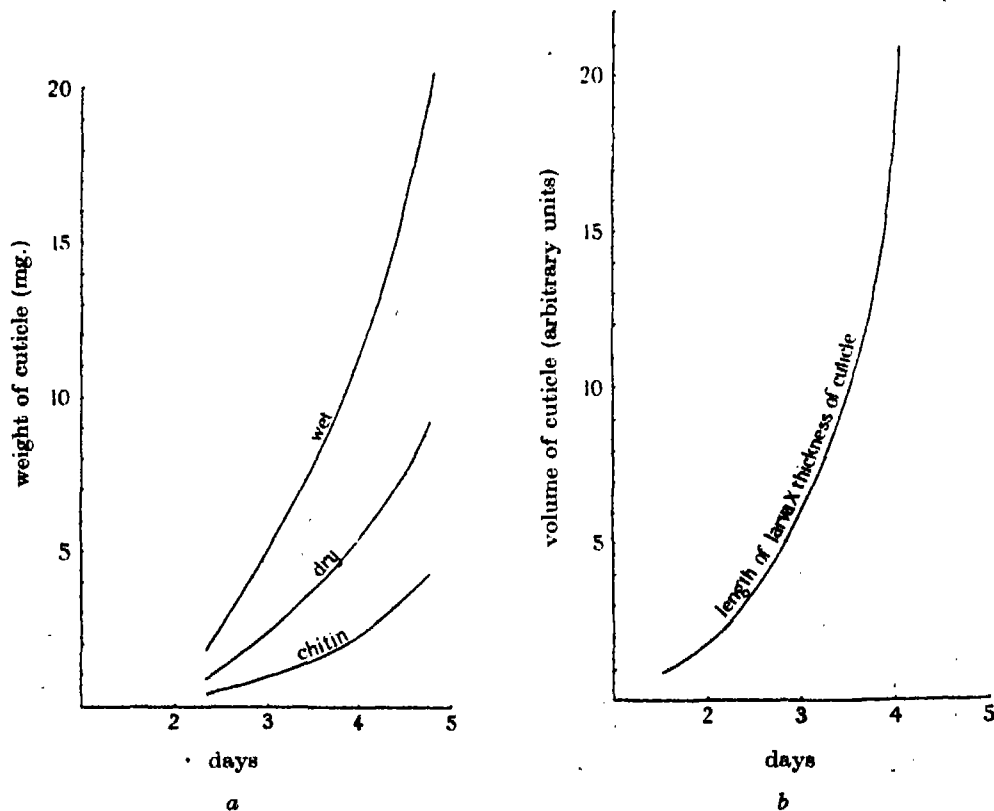


FIGURE 4. *a*. The growth in weight of the larval cuticle. *b*. The increase in volume of the larval cuticle.

In view of the significance of these findings further observations have been made on cuticles scraped clean under water. These showed a lower dry weight, and an apparent water content of 70%, agreeing with the figure given by Fraenkel & Rudall, but again no deviation from the progressive mode of increase represented in figure 4*a* was noted.

The information resulting from a study of the changes in cuticle thickness on the one hand, and of weight increase on the other, remain irreconcilable until the growth of the cuticle is considered in relation to the growth of the larva as a whole.

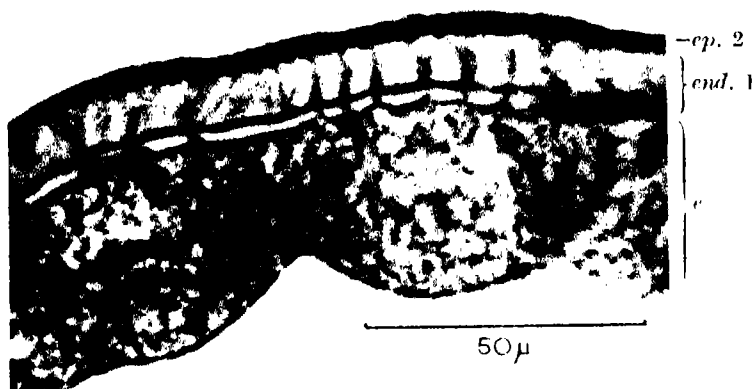


FIGURE 5

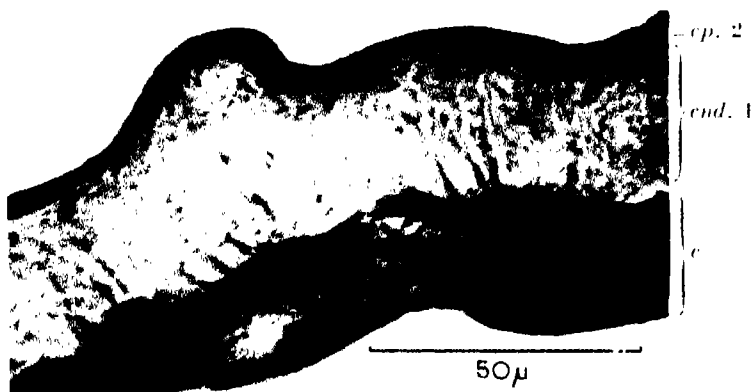


FIGURE 6



FIGURE 7

Then the significance of the sudden increase in cuticle thickness becomes apparent, and it also becomes possible to suggest the cause of the helicoidal form of the pore canals.

As indicated in figure 3 the larva grows rapidly, and reaches its full size at about the end of the third day. During this rapid growth the crop becomes fully distended with food. No further change in size of the larva takes place until the pupal contraction, but during the later part of larval life the crop is progressively emptied.

During growth in length of the larva the area of the cuticle is greatly enlarged and little margin of cuticle secretion is available for increase in its thickness. When growth in length ceases, however, all secretion of cuticle is directed towards increase in thickness, and the full effect of the secretion of new cuticle is seen in sections as a sudden expansion of the cuticle which begins at the end of the third day. It is not necessary, therefore, to postulate any violent change in rate of cuticle secretion to account for this expansion, and as already shown none takes place.

Some reflexion of the rate of volume increase of the cuticle may be obtained by taking into account the length of the larva and the thickness of its cuticle at intervals during growth. In figure 4*b* is indicated the rate of volume increase expressed in arbitrary units obtained by multiplying the length of the larva by the thickness of its cuticle. The resulting product is, in the case of the youngest larva examined, expressed as unity, and later products proportionately. It is true that figures obtained in this way do not take into account the precise shape and proportions of the larva, and that they do not therefore give a strictly accurate indication of the volume of the cuticle, but they do serve to some extent as confirmation of the results of examination of the rate of increase in weight of the cuticle. As is to be expected, the curve expressing volume increase closely resembles that for weight increase, and again no sudden deviation is noted.

It is possible that the great distension of the crop during early life is the prime cause of the extremely rapid growth of the larva, and that during growth the cuticle may undergo mechanical stretching. Certainly a growing larva is much more turgid than an older one in which the crop is emptying, and the results of measurements on the pore canals indicate that stretching does occur. In larvae less than 2 days old the canals were noted, in longitudinal sections, to have an overall breadth at their branched distal ends of about 2μ . In larvae 3 days old the branched ends of the canals occupy about 5μ , indicating a stretching of the cuticle of the order of 250%. During this period the larvae actually increased in length from 9 to 20 mm., an increase of 220%.

The growing endocuticle therefore appears to be laid down under pressure from within transmitted by the body fluid. This pressure is not manifested in a reduction in thickness of the cuticle, for secretion takes place at such a rate that in spite of compression the cuticle continuously increases in thickness. The growth of the endocuticle and pore canals may therefore be pictured as taking place in the

following manner. As new cuticular substance is added to the endocuticle it becomes compressed, and the cytoplasmic filaments of the pore canals, although undergoing an overall extension due to the thickening of the endocuticle, are necessarily involved in this compression and assume a helicoidal form. It is striking that at just that period (2 days, figure 3 A) when growth of the larva is proceeding most rapidly, and when the compression of the cuticle is presumably at its maximum, those parts of the filaments destined to form the coiled middle portions are still unchitinized. The uncoiled distal portions are chitinized early, before marked compression begins, and the uncoiled proximal portions are chitinized as growth in length is completed and compression ceases.

The expansion of the endocuticle when larval growth ceases is reflected in the great extension of the coiled strands of chitin filling the pore canals, which become pulled out like stretched springs. In one series of observations a larva about 3 days old, in which the outer endocuticle was about 50μ thick, showed a pitch of $2-3\mu$ in the coiling of the canals. A later larva of the same brood, about 4 days old, had an outer endocuticle of about 150μ , and its pore canals appeared sinuous, being actually extended coils with a pitch of $8-9\mu$.

It should be emphasized that the growth of the cuticle as the crop is emptied after the third day does not appear to involve the expansion of previously compressed layers merely due to the relaxation of the pressure of the body fluid. In preparing cuticles for sectioning the body fluid is released when the larvae are cut open, but sections such as that illustrated in plate 8, figure 7 still show the coiled form of the pore canals.

After expansion the laminae of the outer endocuticle show no obvious difference in spacing from that seen earlier. They are therefore more numerous, and as they cannot be added to by the addition of layers at the surface of the epidermis, such as would be produced by rhythmic secretion, it seems probable that, as suggested by Richards & Anderson (1942), they really do represent distinct structural components of the cuticle, being perhaps chitin-protein polymers separated by layers of pure chitin. But it should be noted that whereas Richards & Anderson found that the laminae of the cockroach cuticle disappeared after treatment with alkali, those of *Sarcophaga*, even after the violent treatment demanded by the chitosan test, remain still apparent and are visible in the original photograph represented in plate 8, figure 7.

In conclusion, therefore, it seems that during early growth the outer endocuticle may first be added to by accretion. Its later expansion, after it has become separated from the hypodermis by the formation of the inner endocuticle, appears, on the other hand, to be due to growth by intussusception. There is no obstacle to the growth of the inner endocuticle by accretion.

The growth of the cuticle of the larva of *Sarcophaga* differs from that of the silkworm as reported by Kuwana (1933). In the latter a period of growth at the beginning of the fifth instar is separated from later growth by a comparatively quiescent period.

No mitoses have been observed in the epidermis during the rapid growth of the larval cuticle of *Sarcophaga*. Trager (1935) found that during the growth of larvae of *Lucilia sericata* the epidermis increased in area not by cell division but by an increase in cell size.

VI. THE CHEMICAL COMPOSITION OF THE CUTICLE

Some of the staining reactions of the cuticle have already been noted in discussing the pore canals, and Mallory's triple stain has been referred to as providing a valuable means of differentiating between the epicuticle and the underlying endocuticle. With this stain the resemblance of the cuticle to that lining the foregut of the lobster (Yonge, 1932) is very marked, the epicuticle apparently corresponding to the 'cuticle', and the endocuticle to the 'chitin' of *Homarus*. As may be seen from table 1 this correspondence is emphasized when other stains are used, and is largely to be attributed to the fact that the isoelectric points of the constituent layers of the cuticle of *Sarcophaga* agree closely with those obtaining in *Homarus*. By using the methods employed by Yonge (1932) the isoelectric point of the epicuticle was found to lie at pH 5.3, and that of the endocuticle at pH 3.4. Whereas, however, the outer endocuticle consistently showed its isoelectric point at pH 3.4, that of the inner endocuticle proved to be somewhat variable, ranging in different parts of the same cuticle from pH 3.4 to as high as pH 4.3. As in *Homarus*, the differing isoelectric points will be seen to be a reflexion of the differing chemical constitution of the layers of the cuticle.

TABLE 1

	<i>Homarus</i>		<i>Sarcophaga</i>	
	cuticle	chitin	epicuticle	endocuticle
safranin and light green	red	green	red	green
Delafield's haematoxylin	blue	almost colourless	blue	light blue
thionin	blue	colourless	blue in spines	colourless
Mallory's triple stain	red	blue	red	blue
isoelectric point	5.1	3.5	5.3	3.4

Few observations on the isoelectric points of the layers of the Arthropod cuticles are available. Thomas (1944) describes the chitin and cuticle of *Lepas anatifera* as having respectively isoelectric points of pH 5.0 and 3.4-5, but it is evident that in the presentation of his results transposition of these values has occurred, for he remarks emphatically that his figures agree with those found by Yonge (1932) for *Homarus*. In a private communication Professor Yonge states that he agrees that the figures for *Lepas* are wrongly quoted. Browning (1942) describes the isoelectric point of the endocuticula of *Tegenaria* as lying between pH 5.0 and 5.6, but owing to its pigmentation he was not able to determine that of the exocuticula. In *Tegenaria* the endocuticula, in spite of its high isoelectric

point as compared with the chitin of *Homarus* and the endocuticle of *Sarcophaga*, still stains blue in Mallory's triple stain.

In its possession of a thick epicuticle, then, staining red with Mallory but in general showing an avidity for basic dyes, the larva of *Sarcophaga* appears to agree more closely with *Homarus*, and perhaps with Crustacea in general, than it does with an insect such as *Rhodnius*, the epicuticle of which is stated to be less than 1μ thick, not to stain with basic dyes or with lipoid stains, and to be of an amber colour (Wigglesworth 1933). But as will be seen from the results of chemical tests on the cuticle of *Sarcophaga* the epicuticle actually is a double layer, the outer being thin and generally resembling the epicuticle as described for *Rhodnius*, while it is the much thicker inner layer that resembles the 'cuticle' of *Homarus*. Pryor (1940b) affirms that the epicuticle of insects has much in common with the cuticle of Crustacea, but it does not appear that he recognized that the larvae of *Calliphora* and *Sarcophaga* possess a double epicuticle. It is probable that the available accounts of both the insect and the crustacean cuticle are incomplete with regard to the epicuticle, and that the cuticles of these two classes of Arthropods may resemble each other in fundamental structure even more closely than has been previously recognized.

The results of chemical tests on sections and pieces of cuticle (summarized in table 2) abundantly confirm the impression given by the different staining reactions and isoelectric points, that the two layers of the cuticle are of different chemical constitution. They clearly reveal also that the epicuticle is actually a double layer.

TABLE 2

test	outer epicuticle	inner epicuticle	outer endocuticle	inner endocuticle
KOH saturated solution	remains	dissolves	remains	remains
HCl concentrated	remains	slowly dissolves	rapidly dissolves	rapidly dissolves
KOH 5% solution	remains	slowly dissolves	remains	remains
HCl 5% solution	remains	dissolves	slowly dissolves	slowly dissolves
xanthoproteic reaction	+	+++	++	++
biuret reaction	-	+++	+	+
Millon's reagent	-	+++	++	++
ninhydrin	-	+++	++	+
Mörner's reagent	-	-	+	-
aldehyde reaction	-	-	++	-
chitosan test (Campbell)	-	-	++	+
chitin test (Schultze)	-	-	++	+
Sudan black B	+++	-	-	-
Schultze's reaction	++	-	-	-
argentaftin reaction	+++	-	-	-
ferric chloride test	-	-	-	-
thionin	+	+	-	-
		only in spines		
methylene blue	+	++	+	-

In their differential solubility in acids and alkalis the layers of the cuticle agree with those of *Homarus* (Yonge 1932). In boiling saturated potash solution the thick epicuticle dissolves, but the endocuticle remains, while in cold concentrated HCl the epicuticle is more resistant than the endocuticle. These differences are chiefly due to the distribution of protein and chitin in the cuticle. The same general results are given when more dilute solutions are used, but the greater resistance of the epicuticle seen with concentrated acid is not apparent with a dilute acid solution, the epicuticle actually dissolving more rapidly than the endocuticle.

The epicuticle

Valuable information has been obtained by subjecting pieces of cuticle to the action of dilute acids and alkalis, and after varying periods of treatment sectioning and staining them in Mallory. After treatment with dilute (5%) HCl at 95° C for 24 hr. the endocuticle was considerably macerated but still stained blue. The epicuticle was represented only by an exceedingly thin membrane detached from the remainder of the cuticle, and also staining blue. Cuticles treated similarly but for a shorter period had a similar general appearance, but although the thin blue membrane was present as before it was attached in places to the endocuticle by the remains of the red-staining epicuticle. It was at once apparent that what had until now been regarded as a single thick epicuticle possessed in fact a thin surface layer of different constitution. The thin outer layer of the epicuticle also resists treatment with potash solution, whether dilute or concentrated, and persists after the thicker inner epicuticle has disappeared.

Re-examination of the thin serial paraffin sections referred to in describing the pore canals revealed the outer layer of the epicuticle as a sharply defined very thin line forming the outer border of the main mass of the epicuticle (figures 1, 2, *ep. 1* and *ep. 2*). The two epicuticular layers differ in staining reaction and are therefore quite distinct. In Mallory-stained preparations the outer layer is blue and the inner thick layer red; with safranin and light green the outer is a clear green and the inner red; and with thionin the outer layer stains deeply, but the inner only feebly or not at all except in the spines of the cuticle. It therefore seems justifiable to refer to the layers as the inner and outer epicuticles, and this procedure is supported, as will be seen later, by the results of chemical tests on the cuticle.

In no sections of normally fixed and treated cuticles was any separation of the two epicuticles encountered, but sometimes the epicuticle as a whole was detached from the endocuticle as a result of mechanical damage to the section, which then presented an appearance similar to that illustrated by Yonge (1932, figure 7). As in *Homarus* it presumably indicates that the epicuticle is in a state of tension. The outer epicuticle is considerably less than 1 μ thick, whereas the inner extends to about 4 μ .

In frozen, hand or paraffin sections stained with Sudan black B (see Lison 1936) the outer epicuticle stains deeply, but the inner becomes at most merely grey. This indication of the presence of lipid substances in the outer epicuticle is

confirmed by application of Schultze's modification of the Liebermann-Burchardt reaction for cholesterol (Lison 1936). The outer epicuticle alone gives a positive reaction. The persistence of the outer epicuticle in cuticles treated with hot concentrated potash solution is perhaps therefore to be explained by the presence in it of unsaponifiable sterols, as well as by the oxidation and polymerization of its lipoids (see later).

The destruction of all layers of the cuticle but the outer epicuticle by cold concentrated HCl affords a ready means of isolating this layer. It remains as a thin transparent membrane, and in spite of the presence of lipoids in it is not dissolved by cold ethyl alcohol, ether, or chloroform. After these solvents it still stains readily with Sudan black. It is therefore not surprising to find that in paraffin sections, the preparation of which involves treatment with lipid solvents, the outer epicuticle is prominently stained with Sudan black or to a less extent with the less effective Sudan III. The outer epicuticle of *Calliphora* stains even more heavily than that of *Sarcophaga*, and appears to be rather thicker.

Pryor (1940b) pictures the epicuticle, which he regards as a single layer, as consisting of a tanned protein membrane impregnated with oxidized and polymerized lipoids. Richards & Anderson (1942) have shown that the epicuticle of the cockroach is a double structure, the outer layer being thinner and more resistant to treatment with acids than the inner. They suggest that an originally single protein epicuticle has become differentiated into 'a thin outer layer of polymerized lipotanned protein and a thicker underlying layer of tanned protein without lipid'. Kühnelt (1928a) detected the presence of cholesterol in the epicuticle and noted the staining of this layer with Sudan III, but it may be recalled that over a century ago Odier (1823) had recognized the presence of a superficial layer containing fat-soluble material. Chibnall, Piper, Pollard, Williams & Sahai (1934) have described the constitution of the primary alcohols, fatty acids and paraffins of insect waxes, and Bergmann (1938) has identified in ether extracts of silkworm exuviae the presence of two major components, first, a mixture of paraffins of the order C_{27} - C_{31} , and secondly, a mixture of esters of *n*-alcohols and acids of the probable order C_{26} - C_{30} . Cholesterol esters of fatty acids were recognized. Yonge (1932) demonstrated the presence of lipoids in the cuticle of *Homarus*, and later (1936) showed their importance in controlling the permeability of the cuticle. Alexander *et al.* (1944a, b) have given valuable evidence, supporting the view of Kühnelt (1928a), that the lipid epicuticle renders the insect cuticle largely impermeable to water, and have shown experimentally the effect of a thin film of beeswax in reducing the permeability to water of a thin celluloid sheet. Ramsay (1935) had already shown the importance of an external film of fatty substance in reducing the evaporation of water from the surface of the cuticle of *Periplaneta*. Wigglesworth (1942, 1944a, b) refers to a layer of wax at the surface of, but outside, the insect epicuticle. He does not, however, describe the constitution of the epicuticle. Earlier (1933) he had described it in *Rhodnius* as being less than 1μ

thick, containing neither protein nor chitin, insoluble in fat solvents, and capable of being isolated by treatment with concentrated HNO_3 . This description would appear to refer to a thin layer resembling the outer epicuticle of *Sarcophaga*. A thicker inner layer, if indeed present in *Rhodnius*, might well be masked, as it is in the puparium of *Sarcophaga*, by the formation of an exocuticle, or not revealed by the staining methods employed.

From the foregoing, then, it appears that lipid substances may occur variously in the insect cuticle. They may form a relatively labile external layer of wax at the surface of the cuticle, they may be closely bound to the surface of the epicuticle so giving rise to a stable thin outer epicuticle, and they may impregnate the inner epicuticle when this layer is tanned. In the larva of *Sarcophaga* there is no appreciable external layer of wax, and the inner epicuticle, which is virtually untanned, is without lipoids. Hurst (1943a), as the result of work on the mode of insecticide penetration, draws a distinction between the 'free' and 'bound' lipoids of the cuticle, stating that 'the protein and lipid components of the insect cuticle are heterogeneous visco-elastic systems comprising chemically linked networks enclosing the more mobile or labile components which may be removed by solvent action'. This description would appear to apply to an insect in which the thick epicuticle is tanned and impregnated with lipoids.

In work on the hardening and darkening of the cuticle evidence pointing to the wide occurrence in insects of an epicuticle consisting of two layers was encountered. Puparia of *Sarcophaga* are completely softened and almost completely bleached by prolonged treatment with cold diaphanol. Mallory-stained sections of treated puparia completely resemble those of the larval cuticle in showing no exocuticle and in revealing a thin outer epicuticle and a thicker inner epicuticle staining with acid fuchsin. Sections of untreated puparia, on the other hand, show no sign of the epicuticular layers, and the amber-coloured exocuticle is unstained. Beneath the exocuticle the endocuticle stains with aniline blue, and between these layers occurs a red-staining zone which lies far within the cuticle and is not to be confused with the inner epicuticle. From a comparison of these sections it is clear that in hardening of the cuticle the protein of the inner epicuticle becomes heavily tanned and indistinguishable from the exocuticle; indeed, as will be described in a later paper, it is in the inner epicuticle that hardening of the cuticle commences owing to the presence of a phenol-oxidase. It is therefore to be suspected that hard cuticles generally may well possess a double epicuticle like that of *Sarcophaga*. Richards & Anderson (1942), it will be remembered, have already demonstrated such an epicuticle in the hard cuticle of the cockroach.

Terga and elytra of *Blaps mucronata*, *Tenebrio molitor*, *T. obscurus*, and *Dermestes vulpinus* have therefore been softened in diaphanol, sectioned, and stained in Mallory. All show, like the softened puparium of *Sarcophaga*, a thick red-staining inner epicuticle, but although a thin outer epicuticle appears also to be present it was not seen with the same clarity as in the much thicker cuticle of *Sarcophaga*. It seems possible therefore that the thin layer designated epicuticle by previous

authors other than Pryor (1940b) and Richards & Anderson (1942) is actually the outer epicuticle.

Pryor (1940b) supports his view that the epicuticle consists basically of tanned protein by pointing to the fact that various tests indicate the presence of aromatic substances in this layer in larvae of *Calliphora*, *Ephestia* and *Tenebrio*. In his illustration (plate 17, figure 4) of the result of the argentaffin test on the larval cuticle of *Calliphora* the layer giving a positive reaction can only, on account of its thickness, be the inner epicuticle. The argentaffin test has been repeated on *Calliphora* in the present work, and the result obtained by Pryor confirmed, but the application of the test to *Sarcophaga* has yielded a strong positive result only in the outer epicuticle, the inner showing but little sign of blackening. The view that the inner epicuticle is tanned to a less extent than in *Calliphora* is confirmed by observations incidental to the performance of the xanthoproteic reaction on these cuticles. The action of concentrated HNO_3 on the cuticle of *Sarcophaga* produces a very slight dull green coloration in the inner epicuticle, but in *Calliphora* this layer becomes bright green. The colours are suggestive of those caused by the action of concentrated HNO_3 on catechol, and may well be due to the presence of an aromatic substance in the epicuticle. It should be noted, however, that in spite of these indications of the presence of aromatic substances, at no time before pupation does the ferric chloride test for dihydroxyphenols give positive results. The differences between the epicuticles of *Sarcophaga* and *Calliphora* will be referred to further in a later paper describing the hardening of the cuticle.

With reference to the nomenclature to be applied to the layers of the epicuticle, the outer epicuticle, rich in lipoids, may be referred to as the lipid epicuticle, and the inner, fundamentally a layer of protein, as the protein epicuticle. Even in those cuticles in which the inner epicuticle is secondarily hardened and impregnated with lipoids the terms may justifiably be employed, for it is likely that the manner of association of lipoids with the two layers differs fundamentally. Richards & Anderson (1942) have proposed the term 'mediocuticle' for the inner layer of the epicuticle.

Proteins

In examining the constitution of the larval cuticle of *Sarcophaga*, colour reactions indicative of the presence of proteins have been carried out either on pieces of cuticle which were later sectioned, or directly on hand-sections. The general results of these tests are expressed in table 2, and do not call for detailed comment. The positive response of the inner epicuticle to the xanthoproteic and biuret tests, and to the application of ninhydrin and Millon's reagent, is to be particularly noted.

The use of Mörner's reagent and the aldehyde reaction (Cole 1933) indicates the presence of tyrosine and tryptophane respectively in the outer endocuticle of the fully formed cuticle. These substances have not been recognized in other layers of the cuticle, and their presence here will be shown in a later paper to be directly related to the impending formation of a hard and dark exocuticle at pupation.

Similar results have been given by tests on the larval cuticle of *Calliphora erythrocephala*, but here tyrosine and tryptophane were noted also in the inner epicuticle of larvae with full crops. Older larvae which have emptied their crops show neither of these substances in the epicuticle. It is likely that just as tyrosine and tryptophane accumulate in the outer endocuticle of *Sarcophaga* prior to its conversion to exocuticle, they accumulate also in the inner epicuticle of *Calliphora* as a preliminary to the tanning of this layer which, as indicated also by the argentaffin test, takes place not at pupation but earlier. The role of tyrosine and tryptophane in hardening and darkening of the cuticle will not be discussed here, but it may be mentioned that Trim (1941) has recorded a decrease in the amount of tyrosine in the cuticles of a brood of *Sarcophaga* larvae on pupation.

The fact that the inner epicuticle of *Sarcophaga* gives a strong positive reaction with Millon's reagent but responds neither to Mörner's test nor to the aldehyde reaction, is perhaps due to the presence of aromatic substances responsible for the very slight tanning undergone by this layer.

Chitin

The chitin tests of Campbell (1929) and Schulze (1922, 1924) yield positive results in both layers of the endocuticle, but chitin is completely absent from the epicuticular layers (table 2). As described earlier these tests have revealed that the contents of the fully formed pore canals are composed of chitin. The differential solubility of the layers of the cuticle in acid and alkaline solutions is at least partially accounted for by the insolubility of chitin in alkaline solutions.

Although an exocuticle is lacking from the general extent of the soft white larval cuticle, the blunt spines possess hard tips of a pale amber colour. They are formed by the tanning of the epicuticular protein as noted by Pryor (1940b) in *Calliphora*, and unlike the remainder of the inner epicuticle give a negative result to the ninhydrin test. Schultze's reaction for cholesterol, however, gives a stronger positive result than elsewhere, and they stain with Sudan black B and with thionin. It is interesting to note that these characteristics are shared with the outer epicuticle which appears also to consist basically of tanned protein.

In the spines of the cuticle and in the outer epicuticle is seen a close relation between tanning of the epicuticular protein and its association with lipid substances. Pryor (1940b) has pointed out that the addition of aromatic groups is calculated to make the cuticle more lipophilic, but states that the exocuticle of the puparium of *Calliphora* does not contain lipid substances. Wigglesworth (1942), however, suggests that lipoids may sometimes extend far into the exocuticle, and may perhaps occur in the endocuticle also. The presence of lipoids has not been recognized in the endocuticle of *Sarcophaga* larvae.

In concluding this account of the larval cuticle of *Sarcophaga* the structural and chemical differences between the inner and outer layers of the endocuticle may be summarized and emphasized. The outer layer possesses pore canals, and in the late larva is rich in tyrosine and tryptophane. It stains readily with methylene

blue. The inner layer possesses none of these qualities, and shows a less constant isoelectric point than the outer layer. It will be shown in a later paper dealing with the formation of the puparium that these differences are largely attributable to the fact that the hardening and darkening of the cuticle involves only the outer endocuticle, the inner remaining completely unchanged.

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EXPLANATION OF PLATE 8

FIGURE 5. The cuticle of an early third instar larva (1½ days after deposition) of *Sarcophaga*. Flemming without acetic, in saline. Mallory. The pore canals are fully occupied with cytoplasm.

FIGURE 6. The larval cuticle at about 2½ days. Helly. Mallory. The distal portions of the filaments are now replaced by chitin.

FIGURE 7. The fully chitinized pore canals of the larval cuticle at about 3 days. KOH, iodine, and H₂SO₄.

ep. 2, inner epicuticle; end. 1, outer endocuticle; e, epidermis.

Figure 3 is reproduced with acknowledgements to the Editors of *Nature*.

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Excitation of the nerve-muscle system in Crustacea

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[Plates 9, 10]

The nature of the action potential and the mechanical response of crustacean muscle is investigated.

If electric shocks of sufficient intensity are applied to the muscle, graded local contractions occur at the cathode. If the intensity of the stimuli is further increased, propagated action potentials, up to 40 mV, are recorded, accompanied by vigorous twitches of the active fibres. The conduction velocity of the muscle impulse is about 20 cm./sec., at 20° C, and its wavelength about 2–3 mm.

The mechanical and electrical responses of the muscle to motor nerve stimulation are local or propagated, depending upon the number and frequency of the nerve impulses.

With single, or low-frequency, motor nerve impulses a negative potential change is recorded in the vicinity of the nerve endings. It spreads decrementally 2–3 mm. along the muscle fibres, and at 17° C rises to a peak in 3 msec. and falls to one half in about 6 msec. Because of its analogy to the junctional potential of curarized vertebrate muscle it will be referred to as 'end-plate potential' (e.p.p.). The spatial characteristics of the e.p.p. provide evidence for a discrete 'focal' innervation of crustacean muscle fibres, similar to that in vertebrates.

In many muscles, with repetitive stimulation, successive e.p.p.'s continue to grow in amplitude for 0.3–0.5 sec. The degree and time course of this 'facilitation' varies greatly in different muscles; depending upon initial size and rate of growth of successive e.p.p.'s, 'fast' and 'slow' systems can be distinguished. At high frequencies (above 100 per sec.), e.p.p.'s sum to a plateau of several times their individual height.

When the e.p.p.'s have grown or summed to a 'threshold' level, propagated spikes are set up. Spikes in individual fibres are usually asynchronous and occur at a lower rate than e.p.p.'s. If the e.p.p. is slightly below 'threshold', abortive spikes are observed.

A prolonged series of e.p.p.'s is associated with a relatively slow maintained contraction of the junctional region. Propagated spikes, on the other hand, are accompanied by quick twitches of the active muscle fibres. This difference is seen clearly by direct inspection of the exposed muscle fibres, but not by recording the overall tension of the muscle.

In many muscles, local junctional responses account for more than 50 % of the maximum observed tension.

Electric recording on the intact animal shows that a good deal of the normal limb muscle activity is based on e.p.p.'s and local contractions. Propagated muscle spikes were seen only during fast and powerful reactions.

The rate of contraction varies with the frequency of motor impulses as a higher than second power function. This relation, and especially the origin of the very slow contraction at low frequency, is discussed. Recruitment of individual muscle fibres plays only a minor role; the main factor is the rate of summation of the local mechanical activation process at the junction. A further factor influencing the speed of contraction is the spatial spread of the active region, which controls the extent of internal elastic shortening of the muscle.

The various links of the neuro-muscular transmission chain are discussed and compared with the analogous processes in vertebrates.

INTRODUCTION

The neuro-muscular system of Crustacea is of general interest for several reasons:

(i) crustacean muscle fibres are supplied with antagonistic nerve fibres, carrying

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excitatory and inhibitory impulses; (ii) in most muscles, action is brought about by prolonged facilitation; (iii) Marmont & Wiersma (1938) recently discovered that, by suitable timing of the inhibitory nerve impulses, complete mechanical relaxation can be produced without noticeable diminution of the muscle action potential. The first two properties are of interest, as their investigation might provide further clues to the mechanisms of inhibition and facilitation at central synapses of higher animals. The third phenomenon deserves close study, as it may throw some light on the events which mediate between muscle action potential and contraction.

In the present paper, experiments are described which deal with the transmission of motor impulses from nerve to muscle and with the nature of the action potential of crustacean muscle.

METHODS

Animals. The experiments were made on sea-water crabs (*Portunus pelagicus* and *Leptograpsus variegatus*) and fresh-water crayfish (*Euastacus serratus*, *nobilis* and *sulcatus*). No essential differences between these species were observed. The animals could be kept in the laboratory for a few weeks; the crabs survived well in oxygenated sea water, while the crayfish were kept in tap water which was renewed every second day.

Nerve-muscle preparations. Most experiments were done on the extensor of the carpopodite, the flexor and extensor of the dactylopodite and the opener and closer of the claw. In some experiments the muscles were exposed, in others the recording leads were inserted through holes in the shell. The extensor of the carpopodite and the opener of the claw have bundles of parallel fibres which are readily accessible after removing the antagonistic muscle. When exposing these muscles, it is important to avoid undue teasing. To expose suitable portions of the flexor and extensor of the dactylus, it was necessary to damage and partly remove a distal portion of the muscle, leaving the more central parts intact.

In some experiments it was necessary to restrict the innervation to a small muscle strip by cutting the nerve branches to the rest of the muscle. For this purpose the preparation was stained with a methylene-blue solution (0.1–0.5 %, soaking for 5–10 min.). Fine nerve branches and connective tissue lying on the muscle surface usually became well visible after staining. The methylene-blue solution did not seem to impair the excitability of the preparation.

Some histological sections were made which indicated that most muscle fibres have a large diameter, sometimes exceeding 1 mm.

Solutions. Nerves and exposed muscles were soaked for 30–60 min. in oxygenated saline. Pantin's (1934) crab solution and Harrevelde's (Harrevelde & Wiersma 1936) crayfish solution were found satisfactory, with some modifications. To the former an extra amount of CaCl_2 (three times) was added, while the latter was diluted by one-third.

Owing to the necessary manipulations and possibly to inadequacy of the saline, some deterioration in the muscle excitability always occurred. In some experiments deterioration was rapid, while in others consistent responses were obtained for several hours.

Electrical apparatus

Stimulation. A Lucas pendulum was used to deliver two or three induction shocks, or to apply a series of thyatron-controlled condenser discharges. A double thyatron stimulator was designed to give pairs of shocks at variable intervals, at frequencies from 10 to about 500 per sec.

Platinum wires were used throughout as stimulating and recording electrodes.

Recording. A resistance-capacity coupled amplifier stabilized by negative feedback was used. Its time constant was 0.2 or 5.0 sec. for half decline. The short time constant was adequate for the present purpose. A single-sweep time-base circuit was triggered by the Lucas pendulum and applied to a gas-focused or hard cathode-ray tube.

Mechanical recording. Contractions were recorded in some preparations by a light tension lever connected to the tip of the dactylopodite and writing on a smoked drum. In other experiments the movement of the dactylopodite was observed against a scale. On exposed muscles and strip preparations, regions of shortening were located by direct observation with a low-power binocular microscope.

RESULTS

To obtain results which are easy to interpret, it was decided, first, to analyse carefully the records from an exposed muscle strip. With the help of this information the responses of the isolated limb were then explored, this preparation being easier to obtain and surviving longer. Finally, the main conclusions were checked on the intact, circulated leg of an immobilized animal.

A. Muscle responses to direct stimulation

For direct stimulation, the extensor muscle of the carpopodite was used, which contains bundles of parallel fibres up to 2 cm. long. The muscle was exposed by carefully removing its antagonist and the large nerve bundles running between the two muscles. Stimulating and recording electrodes were then placed on a strip of fibres, the preparation being covered with a layer of paraffin oil to prevent drying. Single or double induction break shocks were applied, using a cored or coreless coil. The strength of the stimulus was adjusted so that excitation was confined to a narrow strip of muscle fibres. The excitability varied considerably in different preparations. In some muscles the excitability diminished rapidly, and sometimes no propagated spikes could be set up at all; other preparations, however, gave consistent responses for more than 1 hr. There was always a marked tendency to local contraction at the cathode, which was noticeable with current intensities of about half threshold strength and above ('threshold' referring to the conducted spike).

Propagated action potentials are shown in figure 1. They consist of the usual diphasic spike, and are associated with a vigorous twitch. Spike potentials up to 40 mV were observed. By small movements of the recording electrodes along the active muscle bundle, it was established that the impulse started at the cathode and travelled at a uniform rate of about 0.2 m./sec. at 20° C. The wave-length of the spike is approximately 2–3 mm. The small positive potential in the front and wake of the negative spike is due to a well-known effect (Bishop 1937), obtained when leading from an active fibre on the surface of an inactive volume conductor.

Nerve excitation was avoided in these experiments by placing the stimulating leads near the end of the muscle fibres, at the shell, some distance away from the motor nerves. It will be shown in the following section that the muscle response to a single nerve impulse consists of a very different, non-propagated, type of potential.

B. *Muscle responses to nerve stimulation*

Methods. The simplest results were obtained from the exposed extensor of the carpopodite. After staining with methylene blue and by careful dissection under saline, the main nerve was lifted from the muscle tissue, and all fine branches were cut except one supplying a narrow strip of muscle fibres. It is better to use the limb for this experiment than the claw, because the limb muscle is thinner and therefore yields a finer layer of muscle fibres. In this way it was possible to obtain a preparation with a sharp focus of myoneural junctions, similar to those of the frog's sartorius or cat's soleus (Eccles & O'Connor 1939; Katz & Kuffler 1941). The disadvantage of this, otherwise excellent, preparation is its nerve supply (Harreveld & Wiersma 1937), consisting of two motor fibres and one inhibitory fibre which cannot readily be separated. Simultaneous stimulation of the inhibitory fibre introduced complications in some experiments, but these will be dealt with in a later paper. In all the experiments described below, inhibitory impulses were either absent or without appreciable effect, judging from the vigorous mechanical response. As regards the two motor axons, there is little doubt that the responses recorded in figures 2, 3, 5 and 7 (plates 9 and 10) were due to the 'fast' motor system only (see § B (3)). While the presence of the three nerve fibres had no serious effect on the argument, it was satisfactory that the results were fully confirmed on other muscles (opener of claw, extensor and flexor of dactylus) where the motor axons were stimulated separately.

(1) *'End-plate potentials'*

If a single shock is applied to the nerve of the extensor of the carpopodite, a local negative potential change is recorded in the vicinity of the nerve endings. This potential is analogous in every respect to those observed at the junctions of curarized vertebrate muscle and will, therefore, be referred to as 'end-plate potential' (e.p.p.). It spreads decrementally about 2–3 mm. on either side of the junctional region (figure 2), and can sum with successive e.p.p.'s to a much higher

level (figures 4, 6). If the recording electrode is moved a few mm. along the strip, the potential becomes positive; at an intermediate distance a diphasic e.p.p. is often observed, with an initial positive deflexion, later overpowered by the electrotonic spread from the negative focus. This agrees in all details with the e.p.p. recorded from innervated strips of the cat's soleus muscle (Eccles, Katz & Kuffler 1941) and needs no further explanation.

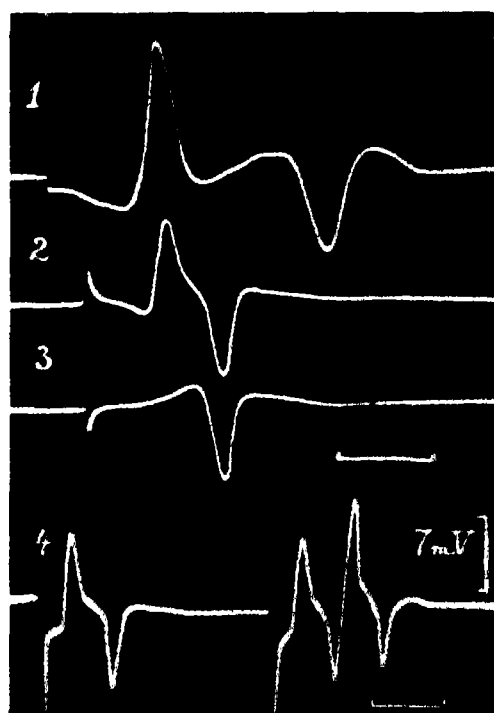
When recording electrodes are inserted at random places through the shell of the isolated limb, the e.p.p. assumes a variety of shapes, mono- or diphasic, depending upon the position of the two electrodes relative to nearby foci of junctions. If one 'active' lead is placed directly over the centre of the muscle and the other lead at some distance away to make it a 'diffuse' or 'indifferent' electrode, one often obtains a simple positive e.p.p. at the active electrode. In this case the active lead actually makes contact with the muscle fibres at, or near, their origin under the shell, i.e. a few mm. away from their junctional region. This was verified by opening the shell and shifting the active lead a few mm. until the negative focus had been located.

The spatial characteristics of the e.p.p. provide definite evidence for a 'focal' innervation of crustacean muscle fibres (see also Holmes 1943). This does not support the concept of a diffuse terminal feltwork of nerves extending over the greater part of the muscle fibres (Wiersma 1941, p. 266). The occurrence, however, of discrete multiple foci similar to those in the frog's satorius (Katz & Kuffler 1941) has not been excluded by the present experiments.

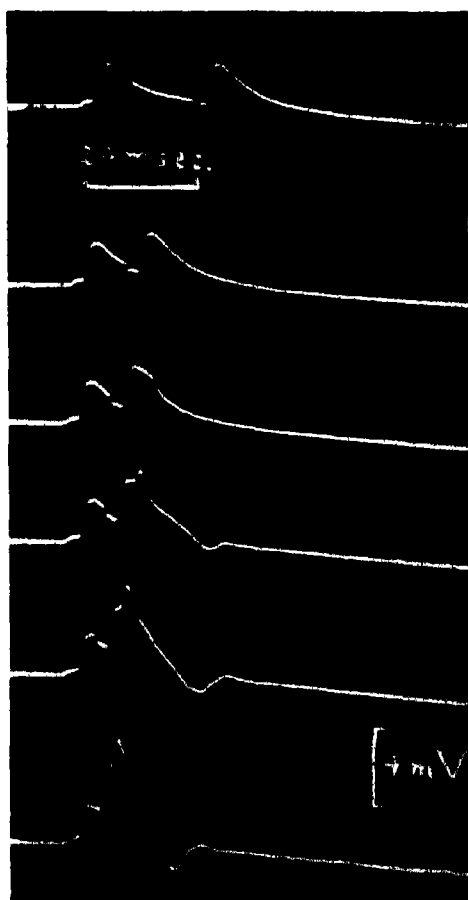
The time course of the e.p.p. at the junctional focus is rather brief: at 17° C it rises to a peak in about 3 msec. and lasts about 25 msec. (half decline in 6 msec.). Often a small spike can be seen preceding the e.p.p. (figure 6(3)). This must be the action potential of the motor nerve, for a similar spike—but without e.p.p.—is obtained when the inhibitor nerve is stimulated. The time relation between intra-muscular nerve spike and e.p.p. is much the same as in vertebrate muscle (Eccles *et al.* 1941): there is no significant delay between the end of the nerve potential and the beginning of the e.p.p.

(2) *Summation with two or more shocks*

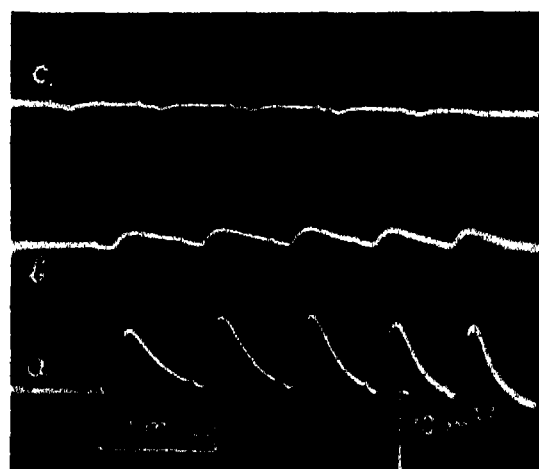
In figures 3–6 the responses of the muscle to multiple motor impulses are shown. The main result is evident: e.p.p.'s are capable of summing to several times their single height and, when a certain threshold level is exceeded, propagated spikes are set up. The advent of a propagated disturbance was marked by the appearance, on top of the e.p.p., of a sharp negative spike, followed after some milliseconds by a positive wave. This positive phase was due to the arrival of the impulse at the second electrode and disappeared when the second lead was made 'indifferent'. Even then a small diphasic dip was usually seen immediately after the initial negative spike. This dip is analogous to the positive phases recorded in figure 1 in the front and wake of the conducted muscle spike, and indicates that the active process has begun to travel away from the junctional focus (Bishop 1937). If,



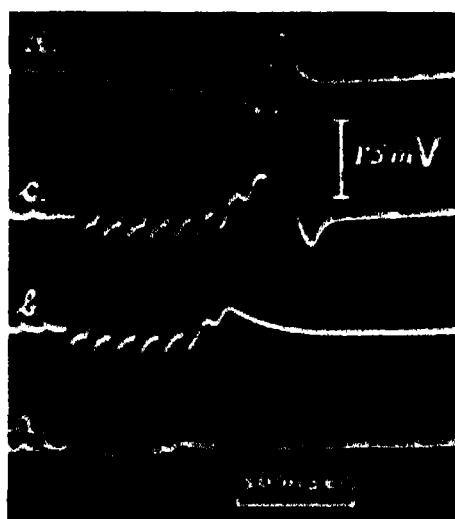
1



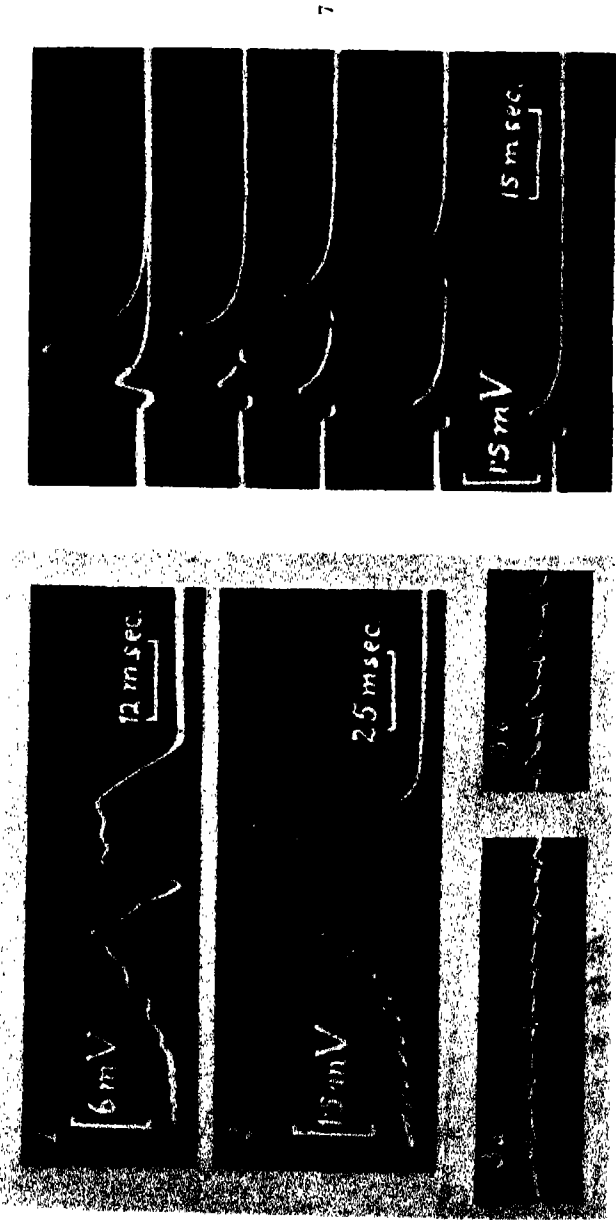
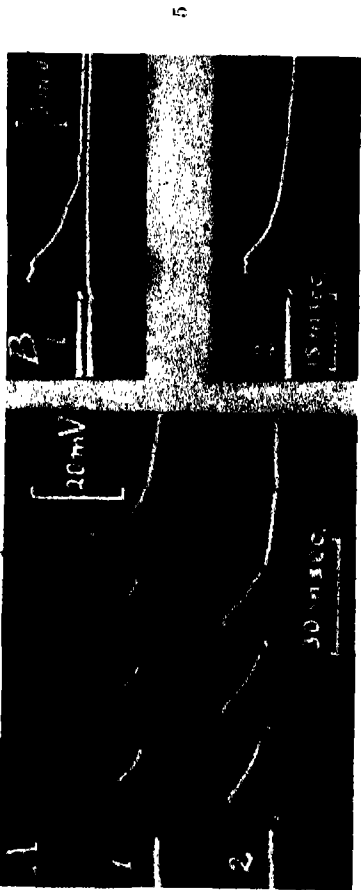
3



2



4



then, the 'active' lead was moved along the muscle strip, a few mm. away from the junction (figure 4d), an initial positive potential was recorded (inverted e.p.p. + positive phase of approaching spike), followed by a negative wave (conducted spike, usually more spread out than at the focus).

Figure 3 shows that abortive spikes occur, at e.p.p. levels slightly below the propagation threshold. These are very similar to the local ('new-born') spike responses in nerve and vertebrate muscle (Hodgkin 1938; Eccles & O'Connor 1941; Kuffler 1942).

Occasionally, even a single e.p.p. was large enough to set up a spike in some muscle fibres (figure 5B). This was seen quite commonly in the closer of the claw, only exceptionally in the extensor of the carpopodite, and not at all in the flexor and extensor of the dactylopodite.

The closer of the claw, as previously shown by Pantin (1936b) and Harrovel & Wiersma (1936) and confirmed in our experiments, has two distinct motor systems (fast and slow), supplied by separate nerve fibres (see below).

In the fast system, a single nerve impulse usually sets up a large e.p.p. + propagated spike, similar to the potentials in figure 5B. Such potentials had already been obtained by Harrovel & Wiersma (1936, figure 5), but their significance was not fully recognized at that time.

(3) Facilitation of end-plate potentials

If a series of shocks at, for example, 50 per sec. is applied to the motor nerve of the opener of the claw, the individual e.p.p.'s grow from a very small initial size to a steady amplitude which may be as much as five times greater. This growth, of 'facilitation', occurs frequently in a 'sigmoid' manner, being very slow during the first few impulses, then becoming faster and eventually approaching a maximum. At 20° C the final amplitude is usually reached within about 0.3 sec. (15 impulses at 50 per sec.); in some opener muscles only six or seven stimuli are needed, while in others stimulation has to be continued for 2 or 3 sec. to complete the growth. Examples are shown in figures 4 and 6. As a consequence of facilitation, the final size of the e.p.p.'s increases with frequency. Raising the frequency from 10 to 70 per sec. usually causes a four- to fivefold increase in the amplitude of the 'grown-up' e.p.p.'s.

This behaviour varies greatly in different types of nerve-muscle preparations. In some muscles, the initial amplitude of the e.p.p. is large, and there is little, or only brief, facilitation; this will be called a 'fast' system. In others the initial e.p.p. is minute, and many stimuli are required to make it grow to a measurable size; this will be referred to as a 'slow' system. (Note: The terms 'fast' and 'slow' apply only to the speed of growth of the e.p.p.'s, not necessarily to the speed of contraction; cf. § 5.)

As pointed out by Wiersma (1941), some muscles are supplied by two or more motor axons with quite distinct synaptic action, one being of the 'fast', the other(s) of the 'slow' type. At one extreme end there is the 'twitch-system' of the closer of the claw (§ B (2)), in which the first muscle potential is a large e.p.p. with superimposed spike, and subsequent potentials rapidly *diminish* in size. At the other end there is the opener of the claw, or extensor of the dactylus exhibiting

a typical 'slow' system. Between these extremes, all transitions and all degrees of e.p.p. facilitation have been observed (cf. Katz 1936*a*, figure 1; Harrevelde & Wiersma 1936, figure 8).

The responses of the carpopodite extensor, shown in figures 2 and 5, were of the fast type, the amplitude of the e.p.p.'s at 50 per sec. staying approximately constant from the first or second impulse. In this system, because of the large size of the single e.p.p., the time factor of facilitation can be studied by applying two shocks at various intervals (figures 3, 7). In some experiments (figure 3) the second e.p.p. was no larger than the first. In others there was an appreciable increase, but only at short intervals (figure 7): the facilitation effect decayed within about 30 msec. (half decline in 5 msec.). It is clear, then, that repetitive stimulation at 50 per sec. (20 msec. shock interval) can only produce a slight growth, restricted to the first two or three e.p.p.'s.

In the opener of the claw and the extensor and flexor of the dactylus, the time factor of facilitation is much longer, as shown by the much more prolonged growth of e.p.p.'s involving many impulses at 50 per sec. The general properties of such a 'slow' system are summarized in table 1.

TABLE 1. RESPONSES OF TYPICAL 'SLOW' SYSTEM

Extensor of dactylopodite of crab, 20°C		
frequency per sec.	' electrical responses	
	e.p.p.	spike
10	remaining very small; sometimes a slight growth during the initial 5-10 shocks, sometimes no growth at all	nil
50	growing up to moderate size within about 0.3 sec., final amplitude about 3 times larger than at 10 per sec.	few occasional spikes taking off from fully grown-up e.p.p.'s
150 and higher	growth and summation up to a plateau within about 100 msec.; height of plateau 5-10 times greater than final e.p.p.'s at 50 per sec.; with prolonged stimulation, e.p.p. plateau declines	numerous spikes taking off from e.p.p. plateau; often only initial spikes seen
frequency per sec.	mechanical responses	
	overall tension	local inspection of exposed muscle
10	very small, rising very slowly	very slow local contraction
50	still slow, but rate is 60-100 times greater than at 10 per sec.	two distinct types: (i) relatively slow, maintained contraction (local), (ii) occasional fast fibrillation (propagated), superimposed on (i)
150 and higher	fast rise; rate more than 10 times greater than at 50 per sec.; tension not fully maintained; often initial peak seen	fast initial contraction; stronger and more frequent fibrillations superimposed

When the e.p.p.'s have grown up to sufficient height, propagated spikes can be seen to arise from them at irregular intervals. In fresh muscles a few spikes are usually observed at 50 per sec. During the experiment the spike threshold generally increases, and eventually much higher frequencies are needed, and *summation* as well as individual growth of e.p.p.'s is required to set up a propagated spike.

At frequencies above 100 per sec., a fairly large muscle spike is often seen, followed by an e.p.p. plateau (figure 6). The occurrence of such initial spikes is reminiscent of Wedenski inhibition in vertebrate, especially eserinizied frog's, muscle (Eccles *et al.* 1942). Repetitive spikes are often seen to arise from the e.p.p.'s at rather long intervals (100 msec. and more). It was clear that the propagated impulses did not follow the frequency of the e.p.p.'s, but invariably occurred at a much lower rate. This was confirmed by direct observation of the mechanical responses (§ 4).

(4) Local and propagated mechanical responses

The e.p.p.'s of crustacean muscle can give rise to powerful local contractions. This was an unexpected, but very consistent finding. There is, indeed, reason to believe that a good deal of the normal muscle activity of Crustacea proceeds without propagated muscle impulses at all (see below).

The evidence was derived from the following observations: (a) At frequencies up to about 50 per sec., the muscles (flexor and extensor of dactylopodite, opener of claw) develop tension at a low rate in the absence of spikes (cf. table 1). (b) At 50–100 per sec., moderately strong contractions occur which may, or may not, be accompanied by spikes. (c) At higher frequencies, during the e.p.p. plateau which often follows the initial spikes, at least one-half of the maximum tension is maintained without noticeable propagated muscle impulses. (d) Further results were obtained by inspecting the exposed muscle fibres directly with a low-power microscope. In this way a clear difference was seen between two types of mechanical responses, depending upon the presence or absence of conducted spikes.

The most direct observations were made on the innervated strip preparation of the carpopodite extensor. While a single e.p.p. was not accompanied by a detectable contraction, a small local shortening in the vicinity of the nerve endings was usually seen with three to five e.p.p.'s at 30–50 per sec. With continued repetitive stimulation, the local contraction gradually became stronger, the speed of initial shortening increasing with the frequency of stimulation. After reaching a steady state, the local contraction was maintained until stimulation ceased.

Whenever conducted spikes, at low frequency, were seen on the oscilloscope, they were accompanied by distinct, quick twitches in individual fibres, in addition to the maintained local contraction. The twitches in these fibres were asynchronous ('fibrillation') and occurred at a low rate, similarly to the propagated spike potentials described in § B (3).

In the experiments on the carpopodite extensor, excitation of the inhibitory axon cannot always be avoided. When inhibition occurred, the local, slow contraction disappeared, but occasional propagated twitches could still be observed. The reason for this effect will be shown in a later paper dealing with inhibition.

The difference between maintained contraction and rhythmic twitches, and their correlation with e.p.p.'s and spikes respectively, was seen quite clearly also in the exposed flexor of the dactylopodite. In this preparation the inhibitory axon was readily eliminated, and thus any interference by inhibitory impulses was ruled out.

There is a rather striking resemblance between the *electric* responses of crustacean muscle and those of partially curarized vertebrate, especially frog's, muscle. The important difference between the two classes seems to be the presence, in Crustacea, of a distinct *mechanical* response accompanying a series of e.p.p.'s. Even this, however, need not be regarded as an essential difference, for local neuro-muscular contractions *have* been observed in vertebrate muscle (Bremer 1932; Cowan 1940; Feng 1941), especially in eserinated preparations where the e.p.p. is large and prolonged (Eccles *et al.* 1942).

The two types of responses, local and propagated, were seen in all the crustacean muscles examined. It was suspected that the presence, and indeed, preponderance of local, rather than conducted, activity might be an abnormal effect due to the impaired condition of the exposed muscle. Careful checks, therefore, were made (i) on the unexposed muscle of the isolated limb, and (ii) by recording normal muscle activity on the intact, circulated, leg. The results of these experiments fully confirmed the existence of both, local and propagated, muscle responses.

Experiments on the whole animal were made by recording the electric responses of the normal limb muscles to central excitation (caused by pinching the appendages or tapping some part of the body). The animal was immobilized by tying it to a dissection board.

The difficulties in interpreting electric responses recorded through holes in the shell have been pointed out in § B (1). Polarity and shape of the potentials depend upon the position of the two leads relative to junctional foci. Nevertheless, there are characteristic features by which e.p.p.'s and spikes can be distinguished. With any given position of the recording leads, the e.p.p.'s, though dependent in amplitude upon the frequency of motor impulses, have a perfectly regular shape. They can be seen to sum and to give the appearance of an 'incomplete tetanus' (a series of ripples raised above the baseline). Spikes, on the other hand, are seen as brief, transient distortions which suddenly and at irregular intervals break into a series of e.p.p.'s.

When a couple of recording electrodes were inserted into the propodite of a walking limb (crab or crayfish), the following observations were made. In the absence of noticeable limb movements, there was only very slight 'background' activity, consisting of an occasional e.p.p. When a sensory stimulus was applied (e.g. strong tapping of the carapace), groups of e.p.p.'s with occasional spikes were seen, accompanied by vigorous limb movements. If the same kind of stimulus was repeated several times, the reactions became weaker, and movements were then seen without spikes, but still associated with groups of e.p.p.'s.

(5) Slow and fast contraction

It is well known that crustacean limb muscles can give very slow or fast contractions depending upon the frequency of nerve stimulation (Pantin 1936*a*; Katz 1936*b*). The only exception to this rule appears to be the 'twitch-system' of the closer of the claw, in which a fast twitch is set up as the result of a single motor nerve impulse (Harreveld & Wiersma 1936).

The very slow development of tension at low frequencies has been attributed by Pantin (1936*a*) and Katz (1936*b*) to a gradual recruitment of muscle fibres analogous to the facilitation in partly curarized vertebrate muscle. The present experiments show that recruitment of muscle fibres, which involves propagated spikes in an increasing number of fibres, plays only a minor part, and occurs only at fairly high frequencies (§ B (4)). The main factor controlling the speed of contraction in crustacean muscle is the frequency and intensity of the local responses in the vicinity of the junctions (see discussion).

The previous observations on the relation between (i) frequency of stimulation and (ii) rate of tension development were readily confirmed and need no further description. The change from slow to fast contraction is gradual, the rate of tension rise being a power function (higher than square) of the frequency. When the mechanical response of the whole muscle is recorded, there is no abrupt change to indicate the appearance of spikes at frequencies of 50–100 per sec. (see, however, § B (4)). This could hardly be expected as spikes are not brought in simultaneously, or at the same frequency, in all fibres. It was found in many experiments that slow *and* fast contractions can be set up by e.p.p.'s alone without spikes. It is equally clear, however, that the initial spikes at frequencies over 100 per sec. contribute greatly to the very quick rise of tension, and are probably responsible for the initial tension peak which is often seen at these high frequencies (Pantin 1934; Katz 1936*b*).

In the 'twitch-system' of the closer muscle, a single nerve impulse gives rise to a large e.p.p. and to fully propagated impulses in many muscle fibres. The contraction is a fast twitch, and if repetitive shocks are applied at, say, 10 per sec., a strong incomplete tetanus occurs, instead of the very slow, fused contraction seen in all the other muscles. If the stimuli are continued, the e.p.p.'s gradually diminish in size, and the propagated spikes disappear. Simultaneously, the twitches become smaller, but are still appreciable when propagation has ceased.

DISCUSSION

A. Gradation of muscular contractions

Most crustacean muscles are supplied by only one or two motor axons. Yet by varying the frequency and number of nerve impulses Crustacea are capable of an even finer adjustment of their muscle reactions than vertebrates with their ample nerve supply (see also Pantin 1936*a*). The reason is as follows. The response of vertebrate muscle is made up of large fixed 'quanta': the all-or-none twitch of a

whole motor unit is its elementary reaction. Crustacea, however, can regulate the force and speed of their responses in much smaller steps and, moreover, have two different means at their disposal: (i) *local* contraction in each muscle fibre, the intensity of which depends upon rate and size of the e.p.p.'s built up at the junction; (ii) *propagated* impulses which can be set up, at a variable rate, in a variable number of muscle fibres. The gradation of both types of response is controlled by the frequency and number of motor nerve impulses and depends upon facilitation and summation of local processes at the junctions.

In spite of the great differences in innervation and regulation of activity, the fundamental units of response upon which muscle activity is based are common to vertebrates and Crustacea, namely, e.p.p.'s, local and propagated action potentials.

It was seen that crustacean muscle has a marked tendency to local contractions, both at the cathode (§ A) and neuro-muscular junction (§ B (4)). The difference between Crustacea and vertebrates, in this respect, is probably merely quantitative and depends upon the relative 'thresholds' of the membrane and contractile substance. Probably, in all types of muscle tissue, an e.p.p. or a catelectrotonic potential can exert two separate actions (provided it is large enough): it can set up (i) *a propagated disturbance of the muscle membrane*, and (ii) *a local activation of the contractile substance*. Once the action potential propagates, it will cause an intense activation of the contractile substance at each point (cf. Brown 1941). Let us assume that a certain minimum quantity of e.p.p. is needed to activate the contractile substance locally, while some other minimum amplitude of e.p.p. is required to set up a propagated membrane potential. If propagation occurs at a relatively low e.p.p. level—and this seems to be the case in fresh vertebrate muscle—local contractions will not be observed. If, on the other hand, the propagation threshold is relatively high, or is made high by membrane fatigue, accommodation, etc. (relative refractory period, eserized muscle, Wedenski effect), then local neuro-muscular contractions are seen even in vertebrates, as was found by Bremer (1932), Feng (1941) and Cowan (1940). Under similar conditions, graded local contractions are obtained with direct current pulses in frog's muscle (Kuffler 1946).

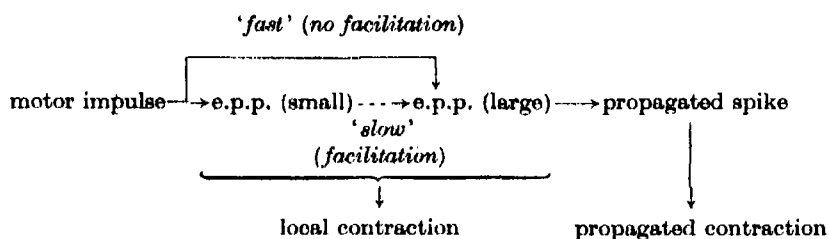
The conclusion that local neuro-muscular responses play a large part in the normal regulation of crustacean muscle activity was checked on the intact animal (§ 4). It appears from those experiments that crustacean muscle fibres are thrown into propagated activity only when fast and powerful actions are required. To execute slow movements or maintain moderate tension, local contractions associated with a series of e.p.p.'s appear to be sufficient.

A special case is the double motor response of the closer of the claw (Harreveld & Wiersma 1936). In this muscle, one motor axon gives rise to large muscle spikes and powerful twitches which rapidly fatigue with repetitive stimulation. The other motor axon sets up small e.p.p.'s which gradually grow in size and are accompanied by a slow, but well-maintained contraction. Harreveld & Wiersma's (1936) work indicates that each of the two motor axons contacts the majority of the muscle

fibres. Hence, by simple 'axon-switching', the same muscle fibres can be thrown into a sudden transient action (propagated), or alternately into a more slowly developing but well-maintained contracted state (local).

B. Neuro-muscular 'transmission chain' in Crustacea

At first sight, neuro-muscular transmission in Crustacea seems to be a very complex process, involving a good many more links than observed in higher animals. A simple scheme is shown below which embodies the main phenomena observed in the present experiments and tentatively puts them into proper sequence:



To this scheme should be added the abortive spikes which are often interposed between the e.p.p.'s and propagated spikes (figure 3), and which are associated with increased local contractions. It is clear that the previous controversial views on the nature of facilitation (Pantin-Katz, v. Wiersma) are deficient; both summation of graded local responses (Wiersma) and facilitation of propagated impulses (Pantin-Katz) occur.

It was pointed out in § B (4) that certain phases of transmission which seem to be so peculiar to crustacean muscle can be retraced in vertebrate, especially frog, muscle, under certain experimental conditions (fatigue, treatment with curare and eserine). In normal vertebrate muscle, the synaptic action of a single motor nerve impulse is so strong that it carries straight through the various phases of the transmission process and leads immediately to an all-or-none response of the muscle. In Crustacea the synaptic effect of a single motor impulse is very weak (except in the 'twitch-system' of the claw), and local summation is required at various stages of the transmission process before a full-size muscle response is obtained. This appears to be more complex, but in actual fact only unfolds a chain of events which, in the higher vertebrates, is compressed into a fraction of a millisecond.

There are a number of outstanding problems which require further elucidation:

(i) *Nature of neuro-muscular transmitter.* The agent which is liberated by the motor impulse and builds up the e.p.p. is not yet known. Previous experiments (Katz 1936b) would seem to rule out acetylcholine and suggest that potassium might play a part in the transmission process. The ineffectiveness of curarine was confirmed on slow and fast nerve-muscle systems (extensor of carpopodite and flexor of dactylus).

(ii) *Inhibition*. No reference is made in the above scheme to the points of action of the inhibitory nerve. This question will be dealt with in detail in a subsequent paper.

(iii) *E.p.p. facilitation*. The gradual growth of individual e.p.p.'s during a series of shocks is similar to, but much more pronounced than, the e.p.p. facilitation in curarized frog's muscle (Schaefer & Haass 1939; Eccles *et al.* 1941). The basis of this facilitation is not known in either case. It must be some process which is long enough to sum during successive motor impulses and facilitates the release or action of the transmitting agent. It is noteworthy that the intramuscular nerve spikes are of constant amplitude, showing no 'staircase' effect (figure 6); but it is possible that a summation of negative after-potentials might play a part in e.p.p. facilitation.

C. *The mechanism of slow contraction*

In *vertebrate* muscle, tension develops at a rate which is quite independent of the frequency of motor impulses; the maximum rate usually occurs with a single isometric twitch. With repetitive stimulation the maximum tension is obtained when the frequency is high enough to give complete tetanic fusion, and nothing is gained by raising the frequency further.

It appears that, in *vertebrate* muscle, a single motor impulse gives rise to a very rapid *maximum* activation of the contractile substance (Hill 1938). The activation process (also known as 'fundamental process' (Gasser & Hill 1924), or ' α -process' (Brown 1941), is brief and lasts little longer than the rising phase of the twitch (Brown 1941). Repetitive stimulation merely *lengthens* the active state but does *not intensify* it.

While this activation is almost instantaneous, the isometric tension develops only gradually. This is due (Hill 1938) to a certain amount of internal shortening of the contractile links against elastic portions of the muscle, tendon and recording system. This shortening must be completed before the full force is registered. By increasing the elasticity of the lever, or by placing a stretch of inactive tissue in series with the muscle, the tension development can be slowed considerably, e.g. by allowing a frog's sartorius an extra elastic shortening of 8 % of its length, the rate of tension rise is reduced to one-half (Katz 1939).

In most *crustacean* muscles the rate of tension development depends entirely upon the frequency of motor impulses. The tension rate is almost nil up to 20 or 30 per sec. and then increases as a fairly high power function (more than square) of the frequency (see also Pantin 1936*a*; Katz 1936*b*).

There are several reasons for this behaviour:

(i) The activation of the contractile substance is a graded local process, and its intensity appears to depend upon the size of the e.p.p. With repetitive stimulation the active state is not only lengthened, but also intensified by *summation*. The rate at which this summation occurs must be proportional to both *frequency* and *size* of the e.p.p.'s; in other words the activation of the contractile substance may be

regarded as an integral of the individual e.p.p. effects until it reaches a ceiling. As the size of the e.p.p. itself, within certain limits, increases almost linearly with frequency, it is not surprising that the speed of contraction becomes a power function of the frequency.

The duration of the activation process, due to a single nerve impulse, is probably only a small fraction of a second. This is indicated by the briefness of the twitch (lasting about 0.5 sec. or less) set up by a short burst of high-frequency motor impulses (200 per sec.), and by the fact that an incomplete tetanus can be obtained if such bursts are repeated at 5–10 per sec. The probable time relations of the various phases of neuro-muscular transmission are shown in figure 8.

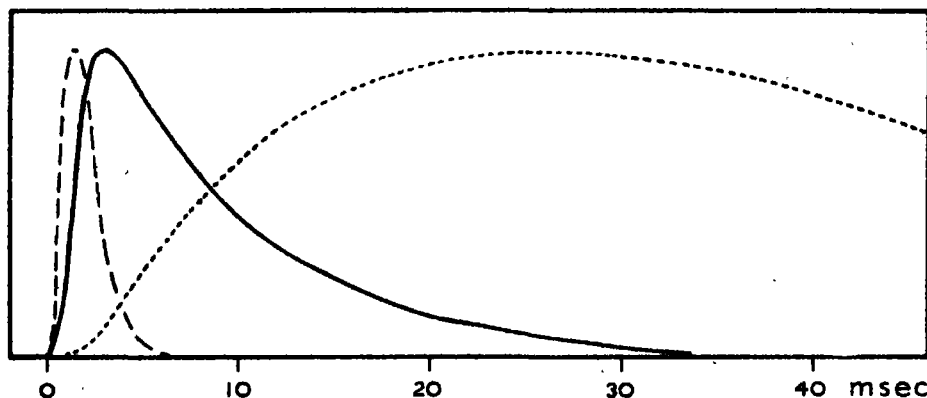


FIGURE 8. Probable time course of events at the junctions, following the arrival of the motor nerve impulse and preceding the development of tension. Full line: e.p.p. Broken line: transmitter action (cf. Eccles *et al.* 1941). Dotted line: mechanical activation process (cf. Brown 1941).

(ii) A further reason for the slowness of the low-frequency contraction is that activation is *local*, and that the 'slackness' of the inactive portions of the muscle has to be overcome before appreciable tension can be registered. In other words, considerable internal shortening occurs which must greatly diminish the rate at which external force can be developed. The situation is illustrated diagrammatically in figure 9 where the combined action of a group of muscle fibres, or fibre bundles, with distributed junctional foci, is considered. Owing to the electrotonic decrement of the e.p.p., a greater portion of the fibre will be activated the greater the e.p.p. Hence, the length of active muscle increases with frequency, and this is a further reason why the rate of tension development must become more rapid. The fastest contractions, of course, are those associated with propagated impulses in the majority of fibres (frequencies above 100 per sec., twitch of the claw).

The internal shortening of small active regions against the inert remainder of the muscle helps to clear up another curious phenomenon. At very low frequencies, e.g. 10 per sec., one might expect an incomplete tetanus, yet the mechanical response is perfectly fused and very slow. At such low frequencies there is little,

and sometimes no, growth of successive e.p.p.'s (table 1), and even the mechanical activation process is probably too brief to give appreciable summation. The tension, however, continues to rise slowly for several seconds before it reaches a steady low level (of the order of 1 % of the maximum tension). This extreme sluggishness may well be due to the fact that only a small portion of the fibre length has been activated.

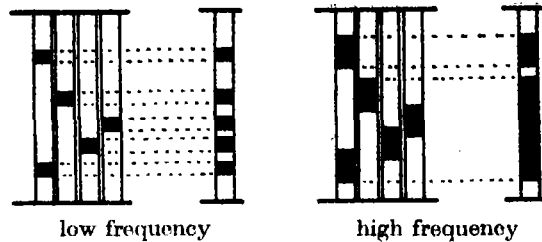


FIGURE 9. Schematic diagram illustrating active (black) and inert (white) regions of the muscle, in the absence of propagated impulses. (1) Low frequency; contraction restricted to narrow junctional regions. (2) Higher frequency: contraction spreads. The combined action of adjacent bundles is represented in the right-hand part of each diagram.

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EXPLANATION OF PLATES

Plate 9

FIGURE 1. Muscle responses to direct stimulation, extensor of carpopodite (crab). Time scale 20 msec. Amplification in record 1 was 30 % less than in records 2-4. (1) 18° C. Distance between recording electrodes 4 mm. (2) 17° C. Distance between leads 2 mm. (3) Same as (2), but first recording electrode was made 'indifferent', leaving only the second wave. (4) 17° C. Two stimuli at 65 msec. interval. Repetitive response to second stimulus. Note: 'Supernormal' size and conduction rate of second response.

FIGURE 2. Strip preparation of extensor of carpopodite (crab). 17° C. Repetitive stimulation at 44 per sec. 'Active' lead at various distances from junctional focus. (a) on focus; (b) 2 mm. away; (c) 3 mm. away. Note: Large size of e.p.p. at focus; initial positive potential at 3 mm.

FIGURE 3. E.p.p.'s and spikes at junctional region. Extensor of carpopodite (crab). Strip preparation. Both electrodes on strip, interelectrode distance 3 mm. 17° C. Intervals between shocks, successively from above: 24, 12, 9.6, 8, 6.4 and 4.8 msec. Note: Propagated spikes in last 3 records taking off at about 3.5 mV. Abortive spike at 9.6 msec. interval.

FIGURE 4. Opener of claw (crab). Strip preparation. 14° C. Stimulation at 160 per sec. (a), (b) and (c) Recording at the junctional region, with increasing number of motor impulses. Spike in (c) arises with the 7th impulse. (d) 'Active' lead moved a few mm. away from the junction. Positive potential preceding negative spike. Note: A block had developed near the recording electrode at (d), and the impulse did not travel past it.

Plate 10

FIGURE 5. 'Fast' system. Extensor of carpopodite (crab). Strip preparations. A. 17° C. Repetitive stimulation at 44 per sec. (1) Pairs of shocks producing large initial spikes rapidly diminishing with continued stimulation. (2) Single shocks, producing pure e.p.p.'s. B. (1) small spike set up by single motor impulse; (2) same as (1) after repeated stimulation. Spike had become 'abortive' and failed to reach second electrode.

FIGURE 6. 'Slow' system. (1) Crab, opener of claw. 18° C. Whole muscle, recording from a junctional focus. Effective stimulation at 160 per sec. Initial spike followed by local 'plateau'. (2) and (3) Crayfish, opener of claw. (2) 16° C. Innervation cut, except to one-third of the muscle. Stimulation at 135 per sec. (3) 14° C. 57 per sec.: (a) initial stimuli, (b) after 0.2 sec. continued stimulation. Note: Intramuscular nerve spike of uniform size precedes e.p.p.

FIGURE 7. Quick facilitation in extensor of carpopodite (crab). 17° C. Shock intervals from above: 5, 8, 16, 24 msec. and single shock.

Histochemistry of the Gram-staining reaction for micro-organisms

BY H. HENRY AND M. STACEY

(Communicated by W. N. Haworth, F.R.S.—Received 12 March 1945)

[Plates 11–12]

Extraction of certain Gram-positive micro-organisms with a 2 % solution of a bile salt (preferably sodium cholate) strips the cell of an essential part of its dye-retaining constituent leaving a Gram-negative cytoskeleton. From the bile-salt extract there was separated a fraction which could be plated back on suitably reduced cytoskeletons and thereby restore in a large measure the Gram-positive character of the cell. The essential agent in the extract was magnesium ribonucleate, and it is considered that the Gram-positive or dye-retaining constituent is a nucleoprotein formed by the combination of ribonucleic acid with a basic protein in the cytoskeleton. The stripping and replating processes could be demonstrated by photomicrographs taken in ultra-violet light.

INTRODUCTION

On 15 November 1883, Friedlander reported the finding of a *Micrococcus* in all but a few of fifty cases of pneumonic lung, and reported the value of a method of staining devised by his colleague, Christian Gram, a Dane. Three months later Gram (1884) recorded the details of the method which bears his name. It would appear that, in dealing with sections of nephritic kidneys, Gram used aniline oil and gentian violet to stain the nuclei blue and followed this up with Lugol's iodine solution in order to get a yellow staining of casts. He found, however, that dehydration with alcohol decolorized the sections completely. On the other hand, similar treatment of sections of pneumonic lung showed micrococci which retained the blue stain. Organisms of a similar character, in that they remained stained after alcoholic extraction, were found by Gram in nineteen cases of croupous pneumonia, nine of pyaemia, one of erysipelas, three of anthrax in mice, and in five cases of tubercle. The presence of organisms which were decolorized in the process could be recognized by using a counterstain such as Bismarck brown, and Gram records that he found typhoid bacilli, among others, to be of this character. Organisms of the first group came to be known as 'Gram-positives', those of the second group as 'Gram-negatives'. Thus there came into being a method of differential staining which gave clear-cut results and which has proved to be of enormous value to bacteriologists ever since that time.

In addition to its use as an indispensable process of bacteriological diagnosis the method can be adapted for the staining of nuclei in general, and it has been shown by Newton (1925) and La Cour (1931) to be an excellent one for chromosomes.

In addition to the Gram reaction there are a number of differential features which distinguish Gram-positives from Gram-negatives. These have been set forth by Churchman (1929*a*) and more recently by Dubos (1941, 1942).

In stained smears of Gram-positive organisms there occur at times individual cells which are Gram-negative. To describe these as senescent or dead cells provides no explanation of their loss of staining capacity. Churchman (1927) found that suspensions of *B. anthracis* lost Gram-positiveness when brought into contact with small amounts of gentian violet, acrid-violet and acrid-flavine. The bacilli became much thinner, their diameter being about 40% less than the normal, and the cell-free filtrates from such preparations were presumed to contain proteins because they gave a positive ninhydrin reaction. On these findings Churchman postulated the existence of a Gram-positive cortex and a Gram-negative medulla. Later, Churchman (1929*b*) was able to produce a similar effect with two strains of *Staphylococcus* by exposing them in aqueous suspension at 52° C for 10 hr. or longer.

We have been able to confirm Churchman's observations in respect of anthrax and *Staphylococcus*, but we interpret them quite differently. We find that the changes he has described are due to autolytic enzyme systems. In fact nearly all the Gram-positive organisms we have so far studied have been found to possess such systems, the potency of which depends upon the selection of a suitable strain and on the carrying out of the experiment at an optimal temperature and at an appropriate pH range. These investigations will be described in a later communication.

It may be recalled too that Gutstein (1924, 1925*a*, 1925*b*, 1926), by means of elaborate staining and mordanting technique, came to the conclusion that the ectoplasmic part of the bacterial cell was responsible for 'Gram-fastness', and that this ectoplasm consisted of a basic ground substance plus an acid lipid.

But more interesting perhaps than the findings of Churchman and Gutstein are the earlier investigations of Deussen (1918), which appear to have been largely overlooked. In his first experiments Deussen dealt with the circumstances which led to the conversion of Gram-positives into Gram-negatives. As this transformation could be effected by acids and alkalis and also by autolysis he concluded that the change is the result of a chemical process. He found, too, that commercial preparations of nuclein and nucleic acid were Gram-fast and lost this property when treated with sodium hydroxide. His tests with tongue epithelium and with sperms showed that here, too, the change from Gram-positive to Gram-negative could be brought about by the same simple chemical means. In his second report Deussen (1921) deals with methods for obtaining Gram-negative cells. Yeast, for example, became almost completely Gram-negative when treated with 2-4% sodium hydroxide. At the end of an extraction the cells were full and round, but on being washed with water four or five times they became very much shrunken, showing that they had lost a large part of their contents. Further, Deussen found that salts of nuclein and nucleic acid diffused through the cytoplasmic membrane of these distorted cells so that they became Gram-positive, while analogous

experiments with albumin, casein, peptone, and lecithin led to negative results. Yoghurt bacilli reacted in the same way as yeast when treated by similar methods. It is unfortunate that the illustrations which accompany Deussen's second paper are disappointing and unconvincing.

An entirely different explanation of the Gram reaction has been put forward by Benians (1912, 1919), who has attributed it to the integrity of the limiting cell membrane. So long as this membrane is intact it does not readily allow the passage through it from within outwards of the large dye-iodine compound which is formed inside the organism in the process of staining. The breaking up of an organism by physical pressure does destroy its entity, as he has claimed, but his further claim that the chemical properties of the resulting amorphous bacterial substance persist unaltered cannot be upheld. That Gram-positive organisms can be converted into Gram-negative debris by crushing is often true enough, but it can readily be shown that this debris becomes Gram-positive if it be treated with a suitable reducing agent.

Striking differences exist in the selective bacteriostatic and bactericidal action of antibacterial agents on Gram-positive and Gram-negative species (see e.g. Dubos 1941, 1942; and also Baker, Harrison & Miller 1941). Thus it appeared to us that a study of the chemistry of the complex in Gram-positives which is responsible for the retention of the basic stain, would be of fundamental importance inasmuch as it might lead to a knowledge of the mode of action of some of the newer bacteriostatic agents such as gramicidin, penicillin, etc.

EXPERIMENTAL

(1) *Bile-salt extraction*

The similarity of autolysis and of bile solubility in the case of the *Pneumococcus* suggested to us the use of bile salt as a means of fractionating the Gram-positive molecular complex.

Our earliest experiments were carried out with a commercial preparation of bile salt termed sodium choleate. When this supply was finished we used sodium glyco-taurocholate. More recently a series of bile salts and their derivatives prepared in this laboratory (in collaboration with Dr F. Smith and Mr M. G. Webb) have been tested, and some of these have been found to be much more useful for our purpose than commercial products.

Washed *Cl. welchii* cells harvested from 4 to 5 l. of an 18 hr. broth culture are suspended in 200 c.c. of 2% bile salt in normal saline. This mixture is poured into a screw-cap bottle of 300 c.c., which is set in a water-bath at 60° C. It is shaken from time to time when the cells show signs of settling down.

The course of 'stripping' of the cells can be followed by removing and examining small samples from the bottle. These samples are centrifuged, the deposits are washed two or three times in saline, and then Gram preparations are made. At the

same time the washed deposits are resuspended in 0.4 % formaldehyde saline and left in this overnight. Thus there are available for the Gram reaction two preparations from each sample—one of the cells washed free of extract and bile salt and the other of these washed cells treated with formaldehyde. Specimens of the first series, which appeared to consist entirely of negatives, often showed a varying number of positive cells after contact with formaldehyde, so that the only reliable indication of change from Gram-positive to Gram-negative is that furnished by washed cells that have been so reduced.

The time taken by bile salt to bring about the maximum of extraction varies from 12 hr. to 5 or 6 days and is dependent on a number of factors. As a rule rough strains are more susceptible than smooth strains, while thin suspensions of cells react more quickly than thick. Of the bile-salt preparations used sodium cholate is the most active.

The method of extracting yeast with bile salt is practically identical with that already described for *Cl. welchii*. Fifty grams of moist yeast are suspended in 200 c.c. of 2 % sodium cholate and kept at 60° C in a screw-capped bottle of 300 c.c. capacity. The course of 'stripping' can be followed by examining small specimens of the suspension from time to time. The transformation from the Gram-positive to the Gram-negative state may occur in from 3 to 36 hr.

Centrifuged deposits from bile-extracted suspensions show in the most successful experiments a mass of Gram-negative cells, but it is nearly always possible to find one or two positive individuals on searching stained microscopical preparations. Where extraction is less complete Gram-positive cells in varying number may be seen scattered throughout the smear. These Gram-positive individuals can be thrown down in the centrifuge as easily as washed normal cells, whereas the extracted Gram-negative cells would seem to be much lighter, for they need much longer centrifuging. It is thus possible to bring about a partial separation of negatives from such positives as may be present in a mixture.

These Gram-negative cells set in formaldehyde saline can be stored in the cold room, and are used for the coupling experiments to be described later.

The bile-salt extract, when separated in the centrifuge, is a translucent yellow fluid which yields a good precipitate with 2½ vol. of alcohol. When dried the precipitate becomes a white or slightly grey amorphous powder, readily soluble in water. Its solutions give a negative or very weak biuret reaction, a strong Molisch test, a positive pentose test with Bial's reagent, and reduce Fehling's solution after acid hydrolysis.

The amount of material removed from cells by bile salt may be considerable. For example, in two 20 l. lots of *Cl. welchii* culture the bile-salt extract of the washed cells gave alcohol precipitates of 1.1 and 1.8 g., while the dried residue of extracted cells was 5.1 and 5.6 g. respectively, so that the amount of material removed from the cells represents between one-fourth and one-fifth of their total dry weight. On the other hand, the amount of bile-salt extract from each 100 g. sample of eight different batches of brewers' yeast varied from 2.0 to 2.46 g. with

an average of 2.32 g., which represents about one-tenth of the dry weight of untreated cells.

In addition to the experiments embodied in this report the bile-salt method has given successful results with the following:

- (1) Anaerobic bacteria: *B. oedematiens*, *B. sporogenes* and *V. septique*.
- (2) Aerobic spore-formers: *B. anthracis*, *B. mycoides*, *B. mesentericum* and *B. megatherium*.
- (3) Certain strains of streptococci and Sarcinae.

(2) *The reconstitution of the Gram-positive molecular complex*

Early in the course of our investigations with *Cl. welchii* it was found that alcoholic precipitation of bile-salt extracts from which cells had not been completely removed gave microscopic preparations of deposits in which there existed considerable percentages of Gram-positive individuals, though these same cells when washed clean of extract proved to be completely Gram-negative. As a result it was found possible to bring about combination of Gram-negative cells, i.e. cytoskeletons, with some constituent of the bile-salt extract so that these became Gram-positive again. It was found, for example, that 1 vol. of washed cytoskeletons with 1 vol. of 1% extract and 1 vol. of alcohol often gave successful results. It soon became apparent, however, that not every batch of extracted cells acted in this fashion and that batches differed greatly in the property of recombining with extract.

Ultimately it was discovered that cytoskeletons became receptive after treatment with suitable reducing agents and that combination occurred by simple contact at room temperature. Sodium sulphite, glyoxal, sodium bisulphite, ascorbic acid, dihydroxymaleic acid, thioglycollic acid and formaldehyde have proved to be effective in rendering bile-salt extracted cells capable of recombining with extract.

The technique is as follows:

Bile-salt extracted Gram-negative cells, well washed in saline, are suspended in saline containing 1% reducing agent and left for 18–48 hr. They are then washed free of the reducing solution, taken up in saline and mixed with an equal volume of 1% alcohol-precipitated bile-salt extract. Smears taken at fixed intervals show that the time necessary for the plating of the cytoskeletons so that these become Gram-positive varies from a few minutes to several hours.

Further coupling experiments showed that Gram-negative yeast cells when treated with a reducing agent became Gram-positive again just as readily with *Cl. welchii* extract as with yeast extract and conversely, the same successful result followed when extracted and reduced *Cl. welchii* cells were thrown into contact with yeast extract. The fact that the extracts are interchangeable suggested not only that they contain the same or closely related substances but also that there may exist a similarity in the chemical structure of that part of the cytoskeletons with which combination occurs.

At this stage in the investigation there became available for coupling experiments a large number of samples resulting from the fractionation of both *Cl. welchii* and yeast extracts. The methods of obtaining these fractions are set out in § (3) of this report. The samples were tested individually for their capacity to restore Gram-positiveness to extracted and reduced Gram-negative cells, and on this basis they could be segregated into two groups, those which gave negative and those which gave positive results. The former proved to be chiefly carbohydrate in character, while the latter were found to give reactions suggestive of nucleic acid.

Thereupon coupling tests were carried out with commercial samples of both the nucleic acids and of such of their derivatives as were obtainable. The following gave completely negative results: adenylic acid, guanylic acid, adenine, guanine, guanosine, guanine sulphate, adenine sulphate, guanine hydrochloride, adenine cytosine dinucleotide and deoxyribonucleic acid.

In fact the only commercial product which gave a positive reaction was the sodium salt of ribonucleic acid. Yet even in this case the coupling occurred more slowly and often with less intense results than with some of our crude bile extracts; so that these last appeared to us to contain either a more strongly coupling nucleic salt or some factor which perhaps accentuates the coupling effect. It was then found that the fractions in the extracts which were most effective in coupling tests contained not only nucleic acid but also magnesium, and on preparing the magnesium salt of ribonucleic acid it was found that receptive cells combine with this salt with great avidity, yielding preparations which stain intensively by the Gram technique.

(3) *The separation and fractionation of bile-salt extracts*

Preparation and fractionation of the extracts was carried out as follows:

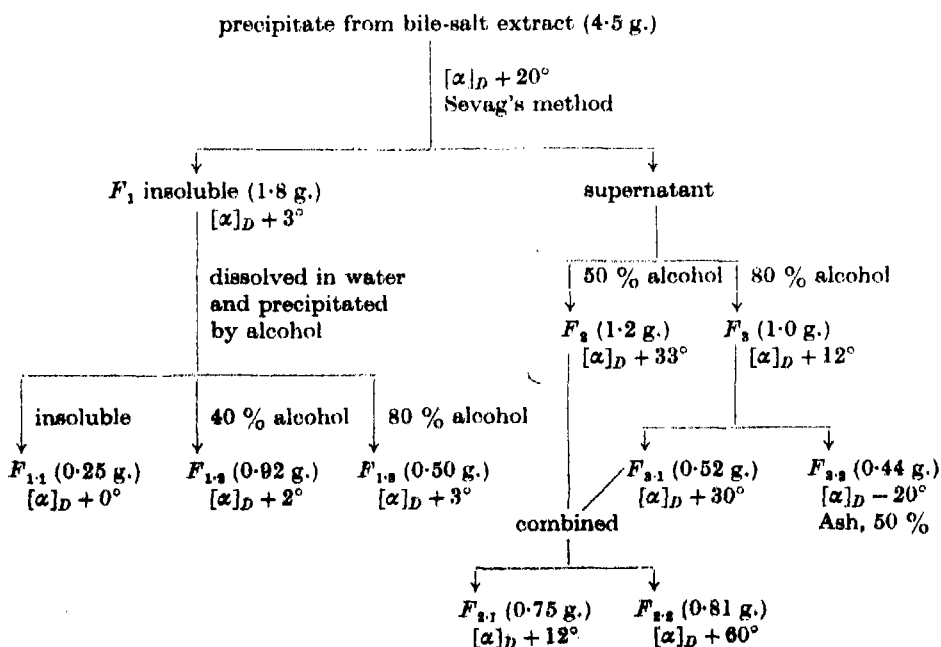
Examination of the bile-salt extract of Cl. welchii cells. The extract was separated from the cytoskeletons by centrifuging for 3 hr. and was concentrated in a vacuum to half-volume. Alcohol ($2\frac{1}{2}$ vol.) was stirred into the solution giving a flocculent precipitate which was collected (centrifuge). This material gave the usual tests for proteins, including biuret, Sakaguchi, etc., for pentose-carbohydrate (Molisch and Bial's) and for deoxypentose (Dische and Feulgen as indicated below) and contained significant amounts of phosphorus. It was dissolved in a small amount of water and acidified with hydrochloric acid when there separated a thick curd-like white precipitate which was immediately collected (centrifuge), washed with alcohol and ether, and dried in a vacuum. Addition of ethyl alcohol to the supernatant solution gave a white precipitate which contained insignificant amounts of phosphorus and pentose and which was shown to consist mainly of a polysaccharide showing $[\alpha]_D^{20} - 20^\circ$ in water ($c = 1.1$).

The phosphorus-containing precipitate was dissolved in 2% sodium acetate solution at pH 8 and then adjusted to pH 4.5 by addition of dilute acetic acid. There separated a small amount of insoluble material, mainly protein, which was removed (centrifuge) and was not further examined at this stage. Addition of

dilute acetic acid to the clear supernatant gave a white precipitate which was purified by being reprecipitated three times by acetic acid in a similar manner and was thus obtained free from protein. It was taken up in 2% sodium acetate and then precipitated by alcohol from solution and was washed with ether and dried. It gave the following analysis: C 39.6%, H 5.8%, N 13.7%, P 9.2%, ash 8.7%, Mg 3.2%, Na 2.0%, and showed a strong absorption band at 2600 Å. It gave all the tests typical of the salt of nucleic acid. It showed pentose tests indicative of the presence of ribonucleic acid and the Feulgen (1919, 1924) and Dische (1930) tests for deoxyribonucleic acid. Quantitative estimations indicated that the fraction contained approx. 90% ribonucleic acid and 10% deoxyribonucleic acid.

Analysis of the cytoskeletons showed the presence in them of relatively large amounts of protein which was specially characterized by giving intense Sakaguchi and biuret tests. A small amount placed on damp, red litmus paper immediately turned the paper blue. The Molisch test was strongly positive and a typical analysis showed P 0.1%, Mg 0.1%, N 11.7%.

Bile-salt extract of Saccharomyces cerevisiae. The bile-salt (sodium glycocholate) solution was evaporated in a vacuum to half-volume and the product in solution, precipitated by adding ethyl alcohol (3 vol.), was isolated and dried. When freshly prepared it was soluble in water showing $[\alpha]_D^{20} + 22^\circ$ ($c = 1.0$), gave weak Sakaguchi and biuret tests, and strong Molisch, pentose, and phosphorus tests. It was dissolved in water at pH 4 and shaken with chloroform according to Sevag's (1934) method. The insoluble precipitate was separated (centrifuge) and the whole fractionated according to the following scheme (all rotations were taken in aqueous solution):



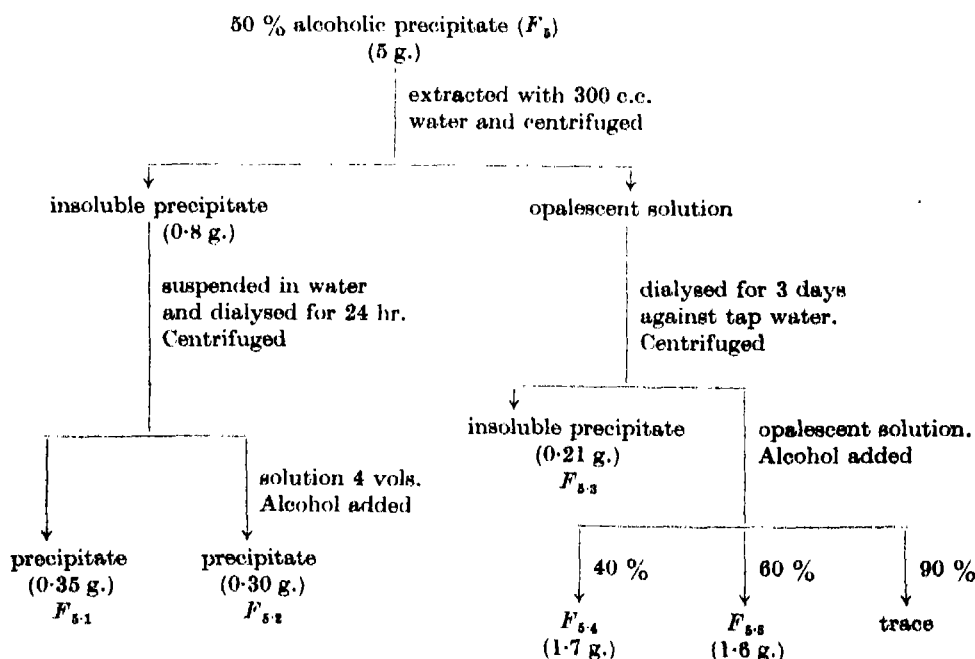
Fractions $F_{1,2}$ and $F_{1,3}$ were those which could be plated back, as described later, on to stripped yeast cytoskeletons, and all the Gram-positive material appeared to have been concentrated in this fraction. It consisted largely of a nucleic acid and a protein (probably in combination).

$F_{2,2}$ was a polysaccharide which could be precipitated by Fehling's solution and consisted mainly of yeast mannan.

$F_{3,2}$ also appeared to consist of a polysaccharide.

Inasmuch as these and further corresponding polysaccharide preparations were inactive in the replating process they were not collected in later fractionations.

In further preparations the use of Sevag's method was discarded when it was found that addition of an equal volume of absolute alcohol to the bile-salt extract gave a fraction which contained all the active material. This fraction contained N and P, and the intensity of its absorption band at 2600 Å indicated that about one-third of it consisted of nucleic acid. A further fractionation was then carried out according to the following scheme:



Fractions $F_{5,4}$ and $F_{5,5}$ could satisfactorily be plated back, as described later, on to reduced cytoskeletons. These two fractions were combined, dissolved in 2% sodium acetate solution and reprecipitated according to the method described for purifying the *Cl. welchii* nucleic acid. The material contained N 13.7%, P 9.2%, Mg 3.2%, and showed $[\alpha]_D^{20} + 6^\circ$ in water ($c = 1.0$). It was protein-free and was completely free from deoxyribonucleic acid.

A typical sample of Gram-negative yeast cytoskeletons had N 11.5 %, P 0.2 %, Mg 0.1 %, and contained considerable amounts of carbohydrate and basic protein material. The Sakaguchi test was vivid.

Isolation of magnesium ribonucleate. A sample of fraction F_5 (3 g.) was carefully dried and extracted by vigorous shaking for 24 hr. with water (100 c.c.) and then centrifuged. To the clear supernatant liquor an equal volume of alcohol was added, and after standing overnight the precipitate was collected (centrifuge). The process was repeated four times until no more insoluble material was present. The product, washed with alcohol and ether, was a white powder (1.1 g.) soluble in water, $[\alpha]_D^{18} + 7^\circ$; P 8.9 %, N 13.5 %, Mg 5.0 %, and gave a strong absorption band at 2600 Å. This material was degraded by the method described by T. J. Bates (1942), and from it was isolated crystalline adenosine and guanine which were further treated to give syrupy *d*-ribose, characterized by formation of the characteristic *p*-bromphenylosazone.

Investigations on commercial yeast nucleic acid. Various specimens of commercial yeast nucleic acid were now fractionated, and samples of calcium, sodium and magnesium salts of ribonucleic acid were prepared. It was shown repeatedly that these preparations could be plated back on to stripped yeast cytoskeletons to restore partially the original Gram-positive character. Magnesium ribonucleate was prepared as follows:

Magnesium ribonucleate from commercial yeast nucleic acid. The sodium salt of commercial yeast nucleic acid was dissolved in water and cautiously acidified with acetic acid to pH 4.5 when a small amount of protein was separated (centrifuge). Dilute hydrochloric acid was now added and the curdy precipitate collected and washed on the centrifuge with water. It was dissolved in faintly alkaline solution, again precipitated with hydrochloric acid and then dialysed through cellophane for 12 hr. The solution was now stirred at 40° for 24 hr. with an excess of magnesium carbonate. The undissolved material was removed, the solution concentrated in a vacuum to small volume and poured into absolute alcohol. The product was washed with ether and dried. It had $[\alpha]_D^{18} + 7^\circ$ ($c = 1.5$) in water, N 13.5 %, P 9.0 %, C 39.0 %, H 6.0 %, Mg 5.2 %, and showed an absorption band at 2600 Å. Material made in this way was used in many replating experiments with various organisms.

Isolation of nucleic acid from B. subtilis. *B. subtilis* cells were extracted with 2 % sodium choleate in the manner described for *Cl. welchii*, and from the centrifuged solution a precipitate was obtained by addition of an equal volume of alcohol. It was reprecipitated several times from faintly alkaline solution by addition of acidified alcohol and was obtained as a white fibrous precipitate free from protein. It had N 11.5 %, P 6.8 %, and gave strong tests indicating the presence of both ribo- and deoxyribonucleic acids. Aqueous solutions of the product were extremely viscous, and the crude nucleic acid appeared to be combined with a polysaccharide residue. The crude nucleic acid could be plated on to yeast cytoskeletons.

Isolation of nucleic acid from Pneumococcus. *Pneumococcus* Type I was grown in broth, allowed to autolyse and then kept in 1% phenol for 24 hr. until quite sterile. The solution was shaken with chloroform and centrifuged. Bacterial 'debris' was precipitated by the chloroform which was removed by washing the precipitate with alcohol. The precipitate was now extracted with bile salt at 60° in the usual way and insoluble material removed (centrifuge). A product was separated from the bile-salt solution by addition of alcohol (2 vol.), and it was purified by being reprecipitated several times from alkaline solution by dilute hydrochloric acid; it was isolated in the usual manner.

The product contained both ribo- and deoxyribo-types of nucleic acid together with basic protein material. Details of these preparations will be presented in a later communication.

(4) *The cytology of normal and of extracted cells*

A. *Cl. welchii* cells. The structure of a bacterial cell may be best described, perhaps, by using the terms which are familiar to the cytologist who deals with plant tissues. The main bulk of the cell, the protoplast, consists of a mass of cytoplasm which must contain the nuclear elements that take part in cell division. Surrounding the protoplast is the cell membrane and outside that the cell wall, on which such a structure as a capsule may be built up. The capsule in the case of *Cl. welchii* may be very obvious if sought in smears of infected tissues, but it does not occur in cultures.

In the Gram reaction the normal *welchii* cell takes on a deep dark blue stain (plate 11, figure 1), and one may assume that the material which takes on the basic dye and holds it is either diffusely distributed throughout the protoplast or is evenly spread on its surface. This dispersal is more obvious with ultra-violet photographs (plate 12, figure 9), where the solid appearance of the Gram staining is represented by an infinity of small discrete granules, tightly packed together.

Cl. welchii cells in process of extraction by bile salt show that the normally dispersed Gram-fast material becomes aggregated in the form of granules which appear to be scattered throughout the protoplast, the substance of which is now Gram-negative (plate 11, figure 2). It is not infrequent to find this material arranged as a string of granules round the perimeter of the cell, an appearance suggesting that it is a surface material. The addition of lithium and lanthanum salts to living *welchii* suspensions produces the same effect of flocculation, and here too the material would seem to be on the surface of the cell.

The granules are very obvious too in ultra-violet photographs (plate 12, fig. 10). In this case they are evenly scattered, and it is not possible to determine whether they are carried on the surface of the cell or are distributed throughout the whole substance of the protoplast. The difference we think may be due to the fact that in Gram preparations the cells are dried and fixed on to a glass surface, whereas the ultra-violet photograph is taken from cells suspended in a fluid medium.

Finally, *Cl. welchii* cells when fully extracted are completely negative by the Gram technique (plate 11, figure 3), yet these same cells show some granulation by ultra-violet light. It may be that the granules are so small as to be easily decolourized by alcohol, or it may be that they represent some material that is not Gram-fast under the conditions of staining.

Cells of *Cl. welchii* which have been extracted with bile salt until they are Gram-negative would seem to be thinner than are unextracted Gram-positive cells (plate 11, figure 3). This effect is in part perhaps due to osmosis, but as the bile extract contains cell constituents which represent from 20 to 25 % of the cell weight it would seem more likely that the diminution in transverse diameter is due to actual loss of substance.

As to the actual location of the nucleic salt, i.e. whether it is a surface material, or whether it is evenly distributed throughout the substance of the protoplast, we have been unable to reach any definite decision.

Extracted *Cl. welchii* cells taken from the same suspension as those depicted in plate 11, figure 3, when placed in 0.5 % Mg nucleate gave good coupling and restitution of the Gram-positive reaction after 20 min. contact (plate 11, figure 4), and it will be noted that they appear ragged as compared with normal unextracted cells.

B. *Saccharomyces cerevisiae*. Healthy yeast cells when fixed by heat and stained by the Gram method appear jet black under the microscope, and it is not possible to make out any intimate detail in such preparations (plate 11, figure 5).

The stripping of these cells by bile salt would appear to occur in two stages. In the first of these the whole cell stains less intensively and takes on varying shades of blue. Soon after this the surface of the cell becomes stippled with blue dots of varying sizes, and less commonly the positive material appears as a network enclosing the whole cell (plate 11, figure 6). When all this surface material is lost it is found that the outline of the cell is no longer apparent in Gram preparations. There are, however, revealed certain details in the internal structure of the cell previously obscured by the positive surface material. The most important of these is a large Gram-positive body circular or oval in shape which we take to be identical with the so-called 'sap vacuole'. In the second stage of stripping, this body is progressively decolourized, the material on its surface and in its substance taking the form of coarse and irregular Gram-positive granules which become fewer in number and smaller in size until they finally disappear. Thus in Gram preparations of completely extracted cells only the framework of the 'sap vacuoles' faintly stained with the counterstain are discernible. It is, however, possible to differentiate the cell outline by establishing a background with nigrosine as in Fleming's (1941) method for capsules (plate 11, figure 7). *

Suspensions of non-extracted yeast when examined by ultra-violet light of 260 m μ show each cell to have a clear cell wall of considerable thickness inside which lies the protoplast. This protoplast is bounded on its outer surface by a cell membrane with which there is incorporated some substance that absorbs ultra-

violet light of 265 m μ (plate 12, figure 11), and only when this is removed by bile extraction does the outline of the 'sap vacuole' as a well-defined structure become obvious (plate 12, figure 13). When the cell contents diminish in volume as the result of osmosis the thin cytoplasmic membrane may become wrinkled as the protoplast shrinks away from the cell wall (plate 12, figure 12), and it will be noted that in this case the cell wall is sufficiently rigid to retain its shape and outline.

In Gram preparations of reconstituted cells (plate 11, figure 8) the positively staining material is found in the form of granules scattered through the substance of the protoplast, and it is aggregated also in and around the vacuole.

It has not been possible so far to obtain preparations showing black Gram-positive cell membranes which are so characteristic of yeast cells before extraction.

Extracted yeast cells (plate 12, figure 13) when coupled with magnesium ribonuclease and photographed in ultra-violet light show that the salt is deposited in and around the vacuole in such a way that this structure seems to be literally smothered (plate 12, figure 14).

When coupling experiments are unsuccessful, failure may be attributed to the use of cells which have suffered autolysis before extraction with bile salt.

DISCUSSION

The *Pneumococcus*, one of the earliest Gram-positive organisms to be described, is possessed of an extremely active autolytic enzyme system and is also readily soluble in bile, features which have been the subject of exhaustive study over several decades. In discussing the facts which were then available Mair (1929) voiced the generally accepted opinion when he stated: 'There is every reason to believe that autolysis and bile solubility are identical and that bile salts act simply by hastening the natural autolytic process.' For our more recent knowledge in respect of pneumococcal autolysis we owe much to Dubos and his colleagues. In 1937 Dubos showed that the breakdown enzyme system contained in all types of *Pneumococcus* operates over an optimal range of pH 6.5 to 8.0, and that the activity of this system is completely destroyed by heating at 60° for 10 min. Also, Dubos & McLeod (1938) in the following year succeeded in isolating from commercial pancreatin and from a diversity of tissues, including human leucocytes, enzymes which were capable of acting on killed pneumococci as substrate. These enzymes are heat resistant, act best at 70° C, and have a pH range of 5.0 to 9.3. Probably they are to be classed with the enzyme discovered by Walter Jones (1920) in pancreas. It has been called a ribonuclease by Dubos (1937) and was investigated by Kunitz (1940) who obtained it in crystalline form. It would appear then that the first enzyme, viz. that obtained from the *Pneumococcus* itself, is inactivated at 60° C, while the members of the second group, which are obtained from animal tissues, are unaffected by that temperature. Moreover, the character of the breakdown enzyme system seems to be different in the two cases. In the first instance the heat-killed pneumococcal cells are first rendered Gram-negative.

They are then disintegrated and finally dissolved. With the second group digestion proceeds no further than the production of Gram-negative cells. It is possible therefore that, by subjecting living cells of *Cl. welchii* and of *S. cerevisiae* to the action of bile salt at 60° C, there may be brought into play a depolymerizing ribonuclease, while the remainder of the bacteriolytic system which produces disintegration and solution at 38° C is suppressed at 60° C. Cells killed by heating to 80 or 100° C we have found can be changed into Gram-negatives by bile salt as easily as living cells. This fact would not exclude the possibility of enzyme action because the known ribonucleases are thermostable and may prove to be highly resistant to heat, especially when they are coupled with the appropriate substrate. At the same time it is well to note that no enzyme resembling a ribonuclease has so far been isolated from bacteria. Perhaps the best argument against the possibility of enzyme action playing any part in bile-salt extraction at 60° C lies in the fact that, whereas bile-extracted cells suitably reduced readily couple up with the magnesium salt to give the Gram-positive molecular complex, cells which become Gram-negative as the result of autolysis will not do so. It would seem that in the latter instance the coupling protein either goes into solution or that, if it remains in the cell, its coupling capacity is lost. At present we prefer to regard the highly surface-active bile salt as dissociating the nucleic acids from their salt-like combination with a protein and then dissolving them.

The magnesium ribonucleate which is found in the bile-salt extract probably exists as a nucleoprotein in the living cell. Whatever the nature of the union between the nucleic acid and the protein it is obviously one which can be readily broken. In fact the ribo-salt can be extracted to a certain extent from Gram-positives by prolonged boiling in neutral physiological saline. As this is also true of meta-chromatinic and volutin granules which have been recognized as nucleic compounds by Meyer (1904) and Zikes (1922), it seems likely that there may be a close kinship or even identity of part of these substances with magnesium ribonucleate. This view is supported by the fact that cells treated with weak formaldehyde resist extraction with bile salt at 60° C, in the same way that granules of volutin after fixation in formaldehyde resist the action of solvents such as boiling water and weak alkali. As coming in the same category mention should be made too of the mitochondria investigated by Regaud (1908) and also of the cytoplasmic particles isolated and analysed by Claude (1941). In both instances the ribonucleoproteins are combined with lipid.

To look upon ribonucleoproteins as being no more than reserve food substances is to miss their possible function as precursors of the deoxy-compounds which go to the fabrication of the nucleus itself. White (1942) has suggested that 'the preliminary stages of mitosis must involve the transference of nucleotides from the cytoplasm to the chromosomes, their conversion from ribo- to deoxyribo-nucleotides, and their polymerization into long chains'. That such a relationship exists is discussed by Mirsky (1943), who cites the experimental evidence of Brachet (1933) in respect of sea-urchin eggs. Before fertilization these were found to contain

an excess of the ribo- over the deoxyribo-compound, but after fertilization the ribo-compound diminishes while the deoxy-compound increases in amount. Caspersson & Schultz (1940) were able to confirm this by their ultra-violet absorption data obtained also from sea-urchin eggs.

Our bile salt-yeast extracts do not appear to contain deoxyribonucleic acid, although Rochlina (1933) and Badian (1937) have demonstrated the presence of Feulgen-staining chromosomes in yeast cells. But these structures are extremely small as compared with the whole yeast cell, and the small amount of deoxy-acid they yield may indeed be masked by a large excess of the ribo-compound. On the other hand, in the case of *Cl. welchii* extracts the deoxy-compound is present in considerable amount, representing about 10% of the total nucleic acid yield. Sevag, Smolens & Lackman (1940) have reported that streptococci yield deoxy-ribonucleic acid in amounts of from 2 to 5% of the total dry weight.

It would appear that in Gram-positives, that part of the cell which retains the basic dye is a type of nucleoprotein, possibly a protein-nucleate. The protein in unautolysed cytoskeletons is firmly bound, contains an arginine constituent and —SH groups, the latter possibly forming part of an oxido-redox system. Further work on the nucleoproteins and on the amounts and ratio of the two nucleic acids in various micro-organisms will be reported in a later communication.

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EXPLANATION OF PLATES

The illustrations of *Cl. welchii* in plate 11 are prepared from direct contact prints taken from negatives on panchromatic material. The bacterial cells are enlarged 2000 diameters, and the differentiation between Gram-positive and Gram-negative individuals has been effected by the use of screens consisting of potassium bichromate solutions, the source of illumination being a Pointolite electric lamp. The illustrations of *Saccharomyces cerevisiae* were obtained under conditions similar to those which have been described for *Cl. welchii*. The illustrations in plate 12, which represent both *Cl. welchii* and *Saccharomyces cerevisiae*, also at a magnification of 2000 diameters, are from negatives taken with the Barnard ultra-violet microscope, using illumination with a wave-length of 265 m μ .

We wish here to express our grateful thanks to Dr C. R. Harington, F.R.S., Director of the National Institute for Medical Research at Hampstead, for granting us facilities in obtaining these illustrations. We would like also to thank the staff of the Optical Department of the Institute for the time, energy and skill they have given so generously in preparing these ultra-violet prints.

PLATE 11

FIGURE 1. Cells of *Cl. welchii*, solidly stained by the Gram method.

FIGURE 2. A stage in the bile-salt extraction of *Cl. welchii*. Many of the Gram-positive cells are markedly granular, while the Gram-negative cells are but slightly stained with the counter-stain and are indistinct.

FIGURE 3. Extracted *Cl. welchii* cells, which have been immersed in 2 % bile salt at 60° for 18 hr. They were then washed twice in physiological saline, and kept for 18 hr. in 0.4 % formaldehyde. The Gram-stained preparation showed an occasional Gram-positive individual, together with a few faintly granular bacteria, but the bulk of the cells were completely Gram-negative.

FIGURE 4. The Gram-negative cells shown in figure 3 were placed in contact with a 0.5 % solution of magnesium ribonucleate for 20 min. at room temperature, and stained preparations showed them now to be strongly Gram-positive.

FIGURE 5. Cells of *Saccharomyces cerevisiae* stained by the Gram method. They appear quite black and show no structural details.

FIGURE 6. Yeast cells in process of extraction by bile salt. The Gram-positive material has been largely removed from the surface of the cells and is represented by no more than a fine stippling. The so-called 'sap vacuoles' are now evident as large deeply staining Gram-positive bodies.

FIGURE 7. The final stage in the extraction by bile salt shows a mass of cells which when stained by the Gram technique show Gram-negative 'sap vacuoles' only while the cell outlines have disappeared. In this preparation the outlines of fully extracted cells have been made visible by the use of nigrosin.

FIGURE 8. Fully extracted cells washed well and set in 0.4 % formaldehyde, then placed in 0.5 % magnesium ribonucleate, regain their Gram-positive character. The surface of the cell becomes stippled and the 'sap vacuole' is heavily stained with the basic dye.

PLATE 12

FIGURES 9-14 are from negatives taken with ultra-violet light at a wave-length of 265 m μ .

FIGURE 9. Cells of *Cl. welchii* before bile-salt extraction. The cells are loaded with material which absorbs the ultra-violet light and appear as opaque rods packed with or covered over with an infinity of extremely fine granules.

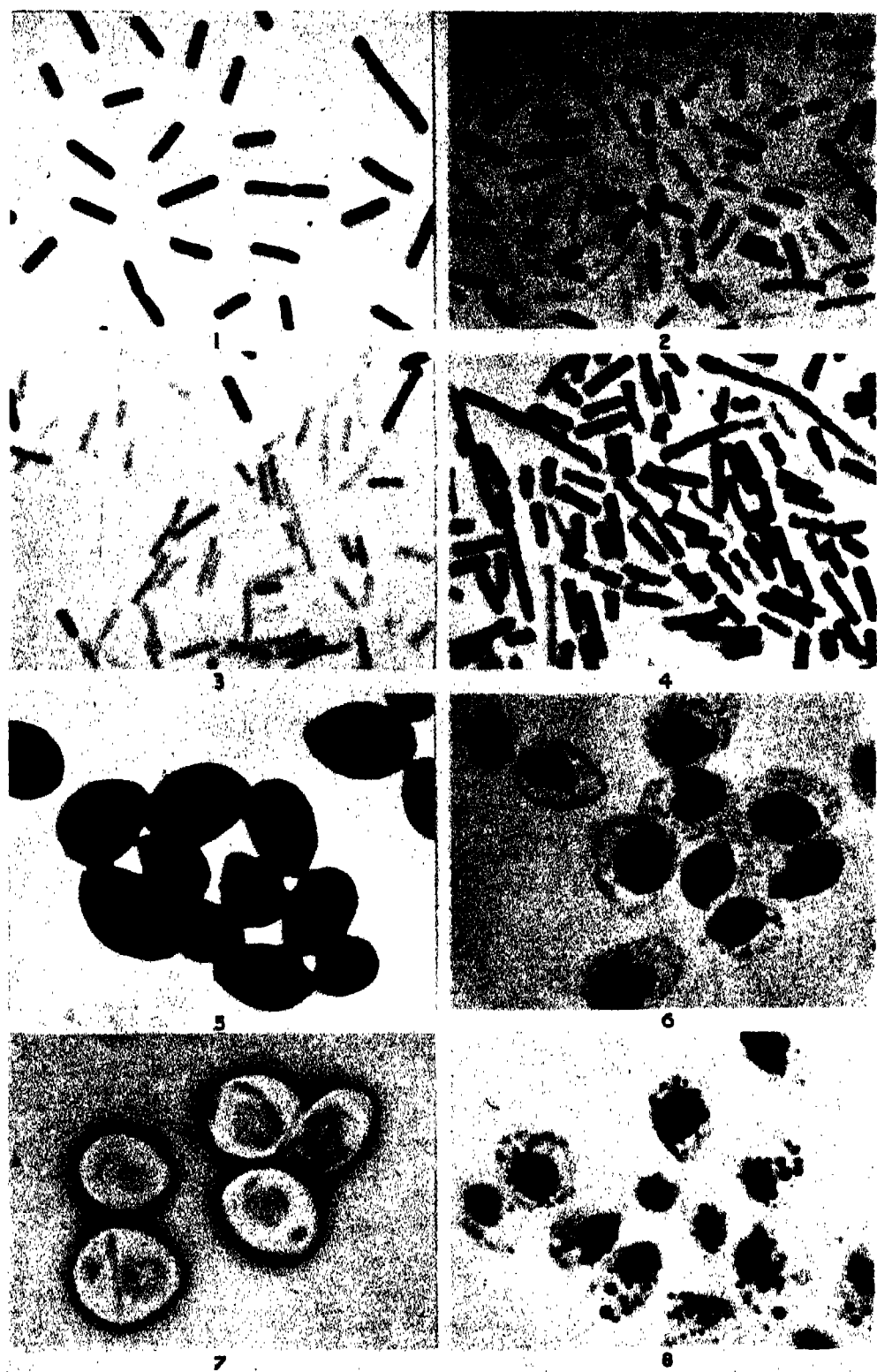
FIGURE 10. Cells of *Cl. welchii* after bile-salt extraction. The ultra-violet absorptive material has been removed except for a few scattered granules, and the cells are now pervious to ultra-violet light.

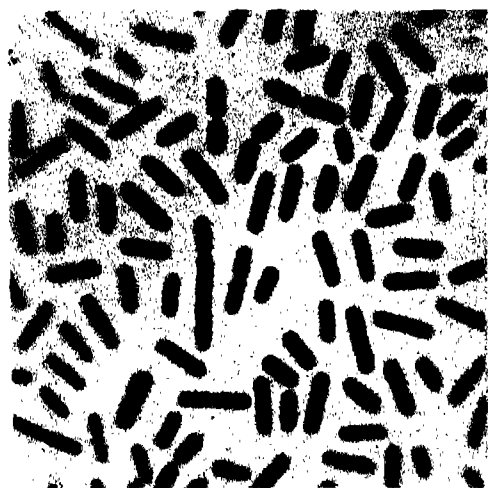
FIGURE 11. Cells of *Saccharomyces cerevisiae* before bile-salt extraction. The absorptive material is aggregated in or on the cytoplasmic membrane, which lies inside the cell wall.

FIGURE 12. Cells of *Saccharomyces cerevisiae* after bile-salt extraction. The absorptive material has been removed, and the cell membranes are wrinkled, though the cell walls are still rigid.

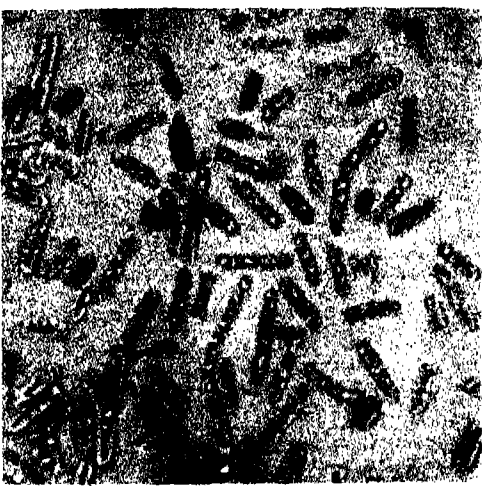
FIGURE 13. Cells of *Saccharomyces cerevisiae* completely extracted with bile salt. The cells are now translucent, and the 'sap vacuoles', also largely translucent, appear as large and sharply defined structures.

FIGURE 14. Bile-extracted cells of *Saccharomyces cerevisiae*, of the same batch as those depicted in figure 5, were treated with 0.4 % formaldehyde saline and then coupled with 0.5 % magnesium ribonucleate. The 'sap vacuoles' are seen to be smothered with absorptive material, and this is present in such large amounts as to fill up the whole cell in certain individuals.

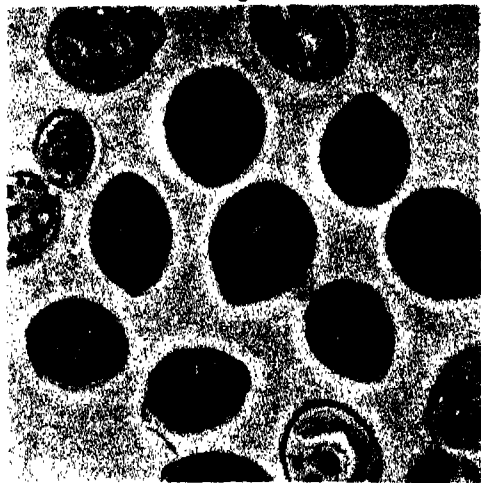




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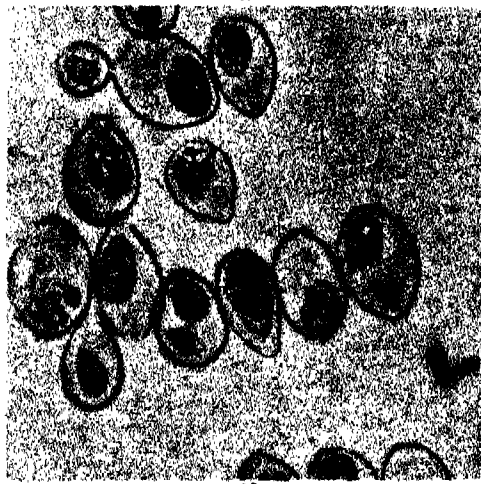
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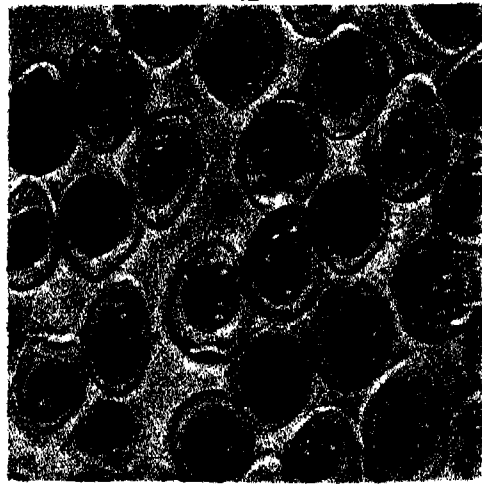
11



12



13



14

The waterproofing process in eggs of *Rhodnius prolixus* Stål

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(Communicated by V. B. Wigglesworth, F.R.S.—Received 7 March 1946)

The seven layers of the chorion covering the egg of the bug, *Rhodnius prolixus*, are all freely permeable to water. The egg has no active physiological mechanism preventing desiccation, and is waterproofed by a layer of wax, less than 0.5μ thick, which covers the inside of the chorion. The wax is similar to that which waterproofs the cuticles of most adult forms of insect, and shows a typical transition point in its water loss/temperature curve at 42.5°C . The waterproofing wax layer is secreted by the maturing oocyte, and is securely attached to the innermost layer of the chorion. The secretion of the wax takes place in the ovary, either just before or after the egg is released from its follicle, but a wax layer can be obtained by incubation of eggs with incomplete chorions. The layer of wax is complete across the inner openings of the micropylar tubes; it is supported at these points on the vitelline membrane before fertilization, and on the fertilization membrane after this has been formed.

INTRODUCTION

In recent studies on the egg of the bug, *Rhodnius prolixus*, Beament (1946*b*, 1946*c*) has shown that the chorion consists of seven layers of proteinaceous material. Each of these layers showed impermeability to specific substances, but no part of the chorion was at all impermeable to water. Beament (1946*a*, 1946*b*) has indicated that other material may be deposited on the inside of the egg-shell, and defined the term 'chorion' as 'that part of the extra-oocytic material which is secreted by the follicle cells'. Since the egg, when laid, shows considerable resistance to desiccation, it is apparent that a waterproofing mechanism exists, and that this is distinct from the chorion.

Previous papers on the physiological characteristics of insect eggs, with special reference to the relation of water loss to temperature and humidity (Johnson 1934, 1940; Clarke 1935; etc.), have produced no evidence of the nature of waterproofing. Evans (1934) indicated that the 'shell' of the egg of *Lucilia sericata* was responsible for most of the waterproofing of the egg, but that some impermeability was retained when the shell was removed, providing that the vitelline membrane was left intact.

However, the observation of Ongaro (1933) is important. Ongaro found that a waxy material, containing approximately 80% paraffins and making up 5.71% of the shell weight, could be removed from the vacated shells of *Bombyx mori* by extracting them with a lipoid solvent. Since Beament (1946*b*) has shown that the chorion layers do not contain free lipoid material, it is possible that the waterproofing mechanism of insect egg-shells may be similar to that of most insect cuticles, i.e. a very thin layer of wax (Wigglesworth 1945; Beament 1945).

It will be the object of this paper to investigate the origin and nature of waterproofing in the *Rhodnius* egg.

METHODS

The rate of water loss of eggs was obtained from their weight before and after desiccation. Eggs were placed on watch-glasses in desiccators with phosphorus pentoxide as the drying agent, and all experiments were carried out with the specimens in the same relative position to the desiccating surface. Weighings were performed in as standardized a manner as possible, after known intervals of time at a standard temperature. Single eggs were weighed on a torsion balance (10 mg./0.02 mg.) and groups of five or more eggs on a chemical balance (200 g./0.1 mg.). In order to check the amount of water adsorbed on to the surface of the egg during weighing, and the disturbance of the atmosphere of the desiccator by frequent removal of specimens, the apparent rate of loss after intervals of 1, 2, 4, 12 and 24 hr. was obtained, using 'waterproofed' eggs, and extrapolated, thus giving the correction necessary for short intervals of desiccation. The rate of water loss is expressed as milligrams of water passing through 1 sq.cm. of the shell area per hour (mg./sq.cm./hr.), and unless stated specifically, the experimental temperature was 25° C. This convention was adopted in order that direct comparisons might be made with similar experiments on the passage of water through insect cuticle which were carried out by Wigglesworth (1945) and Beament (1945). The surface area of the egg was calculated from camera lucida drawings of flattened pieces of shell; no account of the irregular nature of the shell surface has been taken, since the primary water barrier is shown below to be on the smooth inside surface of the shell. Where eggs were freely permeable to water, the figure given expresses the average rate of water loss over the first 15 min. only, since the water content diminishes rapidly.

The formation of the chorion

The paired ovaries of *Rhodnius* are telotrophic, and when each oocyte has accumulated its full content of yolk, it is completely surrounded by a single layer of binucleate follicle cells (Beament 1946b, 1946c). The follicle cells then secrete the chorion layers, consisting of an inner endochorion, and an outer exochorion. The endochorion is composed of five layers:

- A discontinuous *inner polyphenol layer*, made up of islands of tanned protein;
- A *resistant protein layer*, readily penetrated by most water-soluble stains;
- A discontinuous *outer polyphenol layer*;
- An *amber layer*, impermeable to large water-soluble molecules;
- A thick *soft protein layer*.

The exochorion consists of an inner *soft layer* of lipoprotein ('chorionin') and an outer *resistant layer* of a similar material.

All these layers are continuous around the egg, except at the rim of the shell (Beament 1946c), where the pseudomicropylar tubes penetrate from the resistant exochorion layer to the resistant protein layer of the endochorion and the true micropyles form complete open paths through the shell (figure 1).

With the completion of these layers, the egg is released from its follicle and is stored in the lower region of the ovary. The two ovaries may contain up to twenty completed and unfertilized eggs. These are usually laid together, each spending only a few moments in the lower genital region during which time they are fertilized.

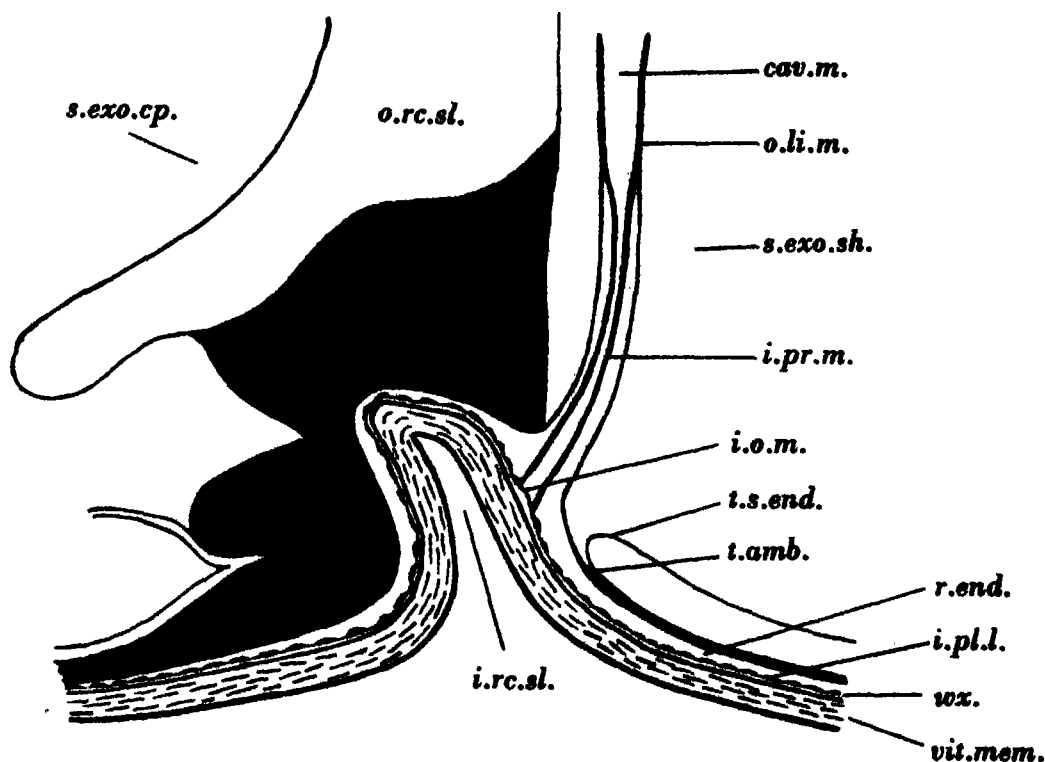


FIGURE 1. Longitudinal section through the rims of the shell and cap, passing through a true micropyle and showing the inner opening of the micropyle (*i.o.m.*) closed by the wax layer resting on the vitelline membrane. *cav.m.* cavity of the micropyle; *i.pl.l.* inner polyphenol layer; *i.pr.m.* inner protein lining of micropyle; *i.rc.sl.* inner recess of the seal; *o.li.m.* outer lipoprotein lining of the micropyle; *o.rc.sl.* outer recess of the seal; *r.end.* resistant endochorion protein layer; *s.exo.cp.* soft exochorion layer of the cap; *s.exo.sh.* soft exochorion layer of the rim of the shell; *t.amb.* termination of the amber layer; *t.s.end.* termination of the soft endochorion protein layer; *vit.mem.* vitelline membrane; *wx.* the waterproofing wax layer. Amber material is shown in black.

Physical and physiological characteristics of eggs

Groups of ten newly waterproofed eggs from the lateral oviducts were desiccated at short intervals of time over phosphorus pentoxide. The desiccator was kept in a thermostatically controlled oven at various temperatures. The change in rate of water loss with increasing temperature is shown in figure 2, where it can be seen that the rate of loss increases very slowly with the initial rise of temperature, but that at a temperature of 42.5° C the permeability of the egg changes abruptly and

further increases in temperature raise the rate of water loss enormously. (It is obvious from the amounts of water lost per hour at higher temperatures that fresh batches of eggs must be used for each new temperature reading. In such experiments the material was kept in desiccators at 25° C for about 4 hr. before use, to ensure that water held in the shell surface, after removal from the ovary, had evaporated.) The shape of the permeability/temperature curve is in every way similar to those obtained by Wigglesworth (1945) in comparable experiments on insects in all stages other than the egg, and by Beament (1945) for the permeability of various membranes covered with waxes extracted from insect cuticles. The waterproofing mechanism of the *Rhodnius* egg may, therefore, take the form of a wax layer with similar physical and chemical characteristics to those which waterproof most insect cuticles; this wax would have a 'transition point' at 42.5° C.

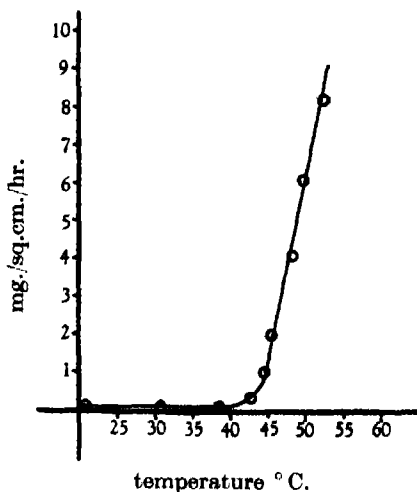


FIGURE 2. Graph showing the relationship of water loss to temperature for freshly water-proofed eggs in a dry atmosphere.

Further details of the relationship between water permeation through the egg and temperature are identical with those for insect cuticle. Newly waterproofed eggs, kept for comparatively long intervals of time at temperatures below the transition point (42.5° C), have normal rates of water loss when transferred to a dry atmosphere at 25° C. The higher the temperature to which eggs are exposed above 42.5° C the greater the rate of loss at 25° C, though this rate is always much lower than the rate of loss above the transition point. Finally, the longer the time interval of exposure at a higher temperature, the greater the water loss at 25° C. For example, eggs exposed to 55° C (by immersion in distilled water) for 2 min. have normal desiccation rates at 25° C, whereas those exposed for 1 hr. are completely dried by subsequent desiccation at 25° C for 24 hr. These results are directly comparable with similar measurements on the permeability of *Rhodnius* nymphs and adults which were made by Wigglesworth (1945).

Eggs within the ovary

Eggs of various stages were removed from the ovaries; their rates of water loss in a dry atmosphere at 25° C were obtained. Eggs with completed chorions which had been released from their follicles were of two distinct kinds. Approximately 80 % of these eggs were 'waterproof' (average rate of loss 0.09 mg./sq.cm./hr.); the remainder were very permeable to water, showing an average rate of water loss of 1.9 mg./sq.cm./hr. over the first 15 min. of desiccation.

Eggs enclosed in follicles again showed two categories of permeability to water. All eggs which had received only endochorion layers were very permeable to water (initial rate of loss 2.3 mg./sq.cm./hr.). These eggs show little difference to the complete but non-waterproof eggs. The majority of eggs with varying amounts of exochorion were very permeable to water (average rate of loss 2.0 mg./sq.cm./hr.), but about 5 % of these had the same order of impermeability as the completed 'waterproof' eggs. Therefore, waterproofing occurs during the deposition of the exochorion, or soon after the egg has been released from its follicle.

Batches of eggs with incomplete shells were carefully removed from their follicles and incubated at 25° C for 3 days in osmotically balanced Ringer's solution. About 90 % of these eggs became normally waterproof, even though they had incomplete shells. Thus, waterproofing would seem to be independent of the activity or presence of follicle cells, and to be carried out by the oocyte without co-ordination with the process of chorion formation.

Sterile, or fertile, waterproof eggs, killed by cyanide fumigation, showed no change in water loss for several days; the waterproofing process is not an active physiological device.

Eggs in the calyx, lateral oviducts and ovarioles proper are sterile (Beament 1946*d*). If removed from these regions and placed in ripe spermatozoa, it is probable that successful fertilization would not take place, as the oocyte is still maturing and cannot be regarded as an ovum. The waterproofing process is *not*, therefore, a part of the fertilization mechanism; it must be considered as a phase in the maturation of the oocyte. Hence waterproofing must involve changes in the surface region of the oocyte, i.e. the vitelline membrane, the inner surface of the chorion, or the innermost layers of the chorion.

The vitelline membrane

After the egg had been waterproofed, the vitelline membrane appeared to have properties identical with the freely permeable membranes found in the earliest stages of chorion formation (and see Beament 1946*b*), and it could not play any part in waterproofing the egg.

The primary wax layer on the unspecialized shell

Before waterproofing, the inner surface of the egg-shell is composed of tanned protein (the resistant endochorion protein layer), partially covered by the granules of the inner polyphenol layer. Dried shells from which the yolk has been removed

have a hydrophobic inner surface owing to the tanned nature of this material (Beament 1946b). Water droplets placed on the inside of the shell do not spread at first, but if the shell is immersed in water for some minutes, the inner surface becomes quite hydrophylic, due, presumably, to the adsorption of water molecules. On the other hand, when freshly waterproofed eggs were taken from the ovaries and treated similarly, the inner surface was extremely hydrofuge, both when dry, and after immersion in cold water for 3 days. No material additional to the inner polyphenol layer could be detected when sections of the shell (cut by a freezing microtome) were observed under the highest magnification of the microscope. If waterproofing is carried out by the deposition of material, it must form an extremely thin layer on the inside of the shell, or else take the form of an impregnation of the inner layers of the shell.

Further evidence was given by injecting water-soluble stains into the shell cavity. These experiments were first carried out on the rear regions of the shell to avoid any anomalies caused by the presence of the inner openings of the micropyles. Freshly waterproofed eggs from the ovaries were placed in Ringer's solution and the caps removed. The yolk contents were washed out at once and the vacated shells placed in a desiccator before filling the shell cavity. This procedure was carried out immediately after removing the caps to avoid the possibility of an artificial fertilization membrane being formed on the inner surface of the shell (Beament 1946d). The dried shells were half filled with stain solution, using a fine glass pipette with micrometer control, and returned to a dry atmosphere. After the stain had dried, the shells were cut longitudinally into halves and immersed in xylene (in which all the stains used were insoluble). It was found that the deposit of stain crumbled off the inside of the shell (see later). The halves were mounted in medicinal paraffin in cavity slides, so that the cut edge of the shell could be observed under the highest power of the microscope.

When freshly waterproofed egg-shells were examined after injection with water-soluble stains in aqueous solutions, there was no colouration whatsoever in the endochorion layers. Solutions used in these experiments included ammoniacal silver nitrate, basic and acid fuchsin, borax carmine, neutral red and iodine, all of which stain the resistant endochorion protein layer when injected into the rear ends of non-waterproofed egg-shells (Beament 1946b). Very great care was exercised during injection, for it was discovered that the slightest abrasion of the inside of the shell by the fine jet of the pipette would allow stain to pass through to the endochorion at that point (although microscopical examination might not reveal any visible damage to the inner polyphenol layer). Where such abrasion took place, the staining properties of the resistant endochorion layers, including the inner polyphenol layer, were identical with those of eggs freely permeable to water. It is, therefore, most unlikely that the waterproofing process involves any *impregnation* of the endochorion layers.

The effect of abrasion on the inside surface of the shell was identical with the effect of similar abrasion on insect cuticle (Wigglesworth 1944, 1945). Inert dusts

(such as fine-grade alumina) rubbed gently over the inside of the shell rendered the inner surface permeable to all the above staining materials; however, dusts placed on the inside of the shell, and left stationary for 24 hr., had no effect on permeability. The material which waterproofs the shell is, therefore, readily abraded, but not adsorbed, by fine dusts (see Wigglesworth 1945; Beament 1945; the cuticular waxes are not affected by static dusts, but are readily disrupted by abrasion).

The yolk contents were removed from freshly waterproofed shells which were then immersed for 1 hr. in cold lipoid solvents such as chloroform, benzene, carbon tetrachloride, etc., making sure that the cavity of each shell was filled with solvent. The shells were then dried and filled with stains as before. After such treatment, the amount of staining in the resistant endochorion was very small; the colour produced, and the depth of penetration, did not compare with that of similarly treated non-waterproofed egg-shells. Lipoid extraction at room temperature does not, therefore, remove all the waterproofing material. On the other hand, extraction of the newly waterproofed shell in *boiling* chloroform, etc., for 6 hr., restored the shell to its non-waterproofed state, and the penetration of stains was identical to their effect on such shells. This extraction does not alter any of the staining properties of the resistant endochorion layers; the waterproofing material must, therefore, lie entirely on the inner surface.

Similarly, waterproofed shells were injected with stains in an aqueous solution containing 5 % of a wax emulsifier known to remove waxes from insect cuticles in a very efficient manner (I.C.I. emulsifier C. 09993; Wigglesworth 1945; Beament 1945). The stain penetrated the endochorion as though the shell had not been waterproofed.

During the development of the embryo, membranes are deposited on the inside of the chorion by the developing egg (Beament 1946*d*). These are left behind when the larva vacates the shell, and form a fragile lining which is completely adherent to the inner surface of the shell. When such vacated shells were cut longitudinally into halves, and immersed in cold chloroform for a few moments, these membranes separated as an almost transparent sheet. This is due, presumably, to the solution of the waterproofing material which appears to act as a cement between the inner polyphenol layer and the membranes. Such a theory is supported by the action of other solvents. Carbon tetrachloride, benzene, ethyl chloride, and xylene acted in a similar manner; absolute alcohol removed the membranes rather more slowly, while acetone required about 5 min. to effect a separation. On the other hand, while 90 % alcohol removed the membranes in about 2 hr., 70 % alcohol loosened them sufficiently to scrape them from the inside only after soaking a shell fragment in the solution for 24 hr. They could not be removed mechanically after several days' immersion in 50 % alcohol, or in water, though shells used in these experiments yielded the membranes readily when dried and transferred to cold chloroform. These experiments are explained by the known solubilities of insect cuticular waxes in various solvents (Beament 1945, 1946*d*).

The thickness of the wax layer

In insects, the thickness of wax on the cuticles so far investigated varies between 0.1 and 0.4μ (Beament 1945). This figure was obtained by extracting the cast skins of insects and calculating the thickness of wax which would be obtained if the lipid extract of the exuviae were spread as an even layer over the surface of the insect. However, although it has been shown (Beament 1946*b*) that no chloroform-soluble material is contained in the chorion, chloroform-soluble material is deposited on the inside of the membranes which are formed during development (Beament 1946*d*). There is some evidence for supposing that this material forms a second wax layer; hence, the term *Primary Wax Layer* is proposed for the waterproofing material at present under discussion. The chloroform extract of hatched shells consists, therefore, of the lipid material of the primary wax layer, contaminated by this secondary waxy material. It was impractical to obtain a sufficient number of freshly waterproofed eggs, and to prepare their shells, completely free of all their contents, for extraction with chloroform. (It was estimated that at least 500 shells would be necessary.)

The total wax from 1000 vacated shells was obtained by repeated extraction of clean shells in boiling chloroform under a reflux condenser. This was weighed, and its volume obtained on a basis of its relative density (Beament 1945). It was then calculated that this wax, spread in a continuous even film over the inside of the shell, would form a layer 0.46μ thick. This figure, therefore, represents the maximum thickness of the primary wax layer. If the wax is distributed evenly between two wax layers, then each would be approximately 0.23μ thick, a figure which is similar to the average thickness of waterproofing wax layers on insect cuticles (Beament 1945). If the thickness were very much greater than this second value, then the wax would certainly be visible as a clear layer in sections prepared so that the material does not come into contact with wax solvents, and with stained layers on either side of it.

The extracted wax was a white material; it was spread on lipid-free butterfly-wing membranes, and the permeability of these membranes at various temperatures was obtained by Beament's (1945) method. This membrane did not show any definite transition point, which supports the suggestion that the extract is a mixture of two waxes.

The closure of the micropyles

Up to this stage it has been established that the primary wax layer lines the whole of the rear portion of the chorion. Similar experiments on the penetration of solvents and stains injected into the front ends of shells showed that wax is also deposited over the inner surface of the neck and cap. It is, however, most important to establish if the wax layer covers the inner openings of the micropylar tubes, which, until waterproofing takes place, give complete access to the surface of the vitelline membrane of the oocyte (Beament 1946*c*).

Over all other parts of the shell, the wax has been deposited on to the rigid inside of the endochorion; it remains on the shell and there is no indication of waxy material attached to the vitelline membrane when the latter is removed. But if wax is also deposited over the inner micropylar orifices, it must exist either as an unsupported film (and less than 0.46μ thick) or by being supported on the vitelline membrane of the oocyte. In this second case it would, presumably, be removed with the vitelline membrane when the shell is prepared for injection.

Freshly waterproofed eggs were taken from the ovaries and the rear ends punctured under Ringer's solution. The yolk contents were washed out, taking great care to remove all the vitelline membrane without abrading the micropylar region. These shells were dried, and, after filling with staining solutions, were again dried. The rim of each shell (containing the micropylar ring) was dissected off and the penetration of the staining materials along the micropyles and pseudo-micropyles was observed. For this purpose the rims were mounted in media in which the particular stain used was insoluble, and without allowing the rim to come into contact with a stain solvent before mounting. Thus, for example, rims treated with oil-soluble materials were mounted direct into glycerol, while those stained with water-soluble stains were placed in medicinal paraffin.

When such shells were filled with basic fuchsin in water, only the true micropyles contained stain; similar results were obtained with ammoniacal silver nitrate and other water-soluble stains. Thus after the removal of the vitelline membrane, the true micropyles are sufficiently open at the inner surface of the shell to allow large water-soluble molecules to pass outwards. When such shells were injected with water-soluble materials after they had been extracted in chloroform, or when stains were applied in water containing 5% of the wax emulsifier C. 00093, both pseudo-micropyles and true micropyles were stained. The wax layer must, therefore, cover the whole of the inside of the rim, with the possible exception of the inner openings of the true micropyles.

However, as soon as the egg is laid and fertilized, a further layer is deposited by the zygote on the inside of the wax. This layer is a thin rigid fertilization membrane (Beament 1946*d*). If the wax layer has been supported on the vitelline membrane up to this time, it would now have this fertilization membrane as its support. It was found that if the injection of water-soluble stains was carried out soon after fertilization, no material passed into the true micropyles from the inside of the shell. The fertilization membrane is permeable to such stains, and it can be concluded that the primary waterproofing layer of wax is complete over the inner openings of the micropyles *after fertilization*.

Further evidence was obtained by immersing whole eggs of various ages in solutions of stains. Waterproof eggs (unfertilized), freshly removed from the ovaries, were immersed for 24 hr. in a solution of basic fuchsin in absolute alcohol. They were then dried rapidly on filter paper and transferred to medicinal paraffin, in which they were burst open, under the binocular microscope. It could be seen at once that the staining solution had penetrated to the yolk which was deep red

in colour. Sections of the shells of these eggs were examined and micropylar rims were dissected from others and mounted in medicinal paraffin. In every egg the only paths that were stained throughout the thickness of the shell were the true micropyles. Thus, after waterproofing, but before fertilization, material capable of dissolving or disrupting a wax layer may pass down the micropyles and into the yolk.

On the other hand, when similar eggs were immersed in aqueous solutions of basic fuchsin, neutral red, etc., no colouration of the yolk ensued. Further examination showed that the linings of the micropyles in these preparations were not stained. Thus lack of penetration to the yolk must be attributed to the inability of the staining solution to wet the linings of the micropyles, rather than to the impermeability of any layer at the bottoms of such tubes. Eggs were therefore immersed in solutions of water-soluble stains made up in 50 % alcohol. It is known (Beament 1946c) that this solution will wet the chorionin and the protein linings of the micropyles, and examination of the shells revealed that the stain had, in fact, penetrated to, and stained the resistant endochorion layer via the bases of the micropyles. It is also indicated above that 50 % alcohol will not soften the primary wax layer sufficiently to release the fertilization membrane, so that unless the wax layer is not complete across the inner openings of the micropyles, no stain will pass into the yolk. It was found that none of the above-listed stains, made up in 50 % alcohol, would penetrate to the yolk, though the solutions must have been in contact with the bottoms of the micropyles for several hours.

When waterproofing wax is deposited by the maturing oocyte, it is present as a thin film across the inner openings of the micropylar tubes, and is supported by the vitelline membrane. After fertilization, the fertilization membrane becomes the supporting substrate for the wax at these points.

DISCUSSION

These experiments leave no doubt that the initial waterproofing mechanism of the *Rhodnius* egg-shell consists of a very thin layer of wax which covers the inside of the chorion. It is, in fact, essentially similar in nature to the mechanism resisting the permeation of water in the cuticle of most insects, including all other life stages of *Rhodnius*. The biological properties of such waxes have been described by Wigglesworth (1945). In the insect cuticle the waxes adhere to the polyphenol layer of the epicuticle (Wigglesworth 1945, 1946); in the egg they are attached to the inner polyphenol layer of the resistant endochorion. Their effect on the permeation of water varies with temperature in a like manner, while the degree of waterproofing conferred by the waxes (at 25° C) is of the same order in both cases (0.08 mg./sq.cm./hr. for *Rhodnius* fifth-stage nymphs; 0.09 mg./sq.cm./hr. for newly waterproofed eggs). Beament (1945) has described the physical and chemical characteristics of cuticular waxes from insects, and the molecular structure which accounts for the extreme water permeability of such thin layers of wax. This is due

to the organization and close packing of the innermost monolayer of wax molecules (cf. Alexander, Kitchener & Briscoe 1944) which are orientated under the influence of the polyphenol substrate on the insect epicuticle.

The waxes probably consist of mixtures of long-chain aliphatic compounds and are similar in composition to beeswax, which contains paraffins, acids and esters (Chibnall, Latner, Williams & Ayre 1934; Chibnall, Piper, Pollard, Williams & Sahai 1934). At the transition temperature (42.5°C for the initial wax layer of *Rhodnius* egg-shells) the mobility of the wax molecules becomes so great that the orientation breaks down, and water passes through much more freely (Beament 1945).

The transition temperatures of these wax mixtures are diagnostic of their physical properties, for Wigglesworth (1945) and Beament (1945) have shown that the higher the transition point, the harder is the wax, and the more waterproof and resistant is the insect. A transition point at 42.5°C is characteristic of a wax which is about midway between those of the least and most resistant insects so far investigated, and is very similar to that of the waterproofing wax of *Pieris brassicae* larvae. The transition point of the cuticular wax of nymphal stages and of adults of *Rhodnius* is 57.5°C . The wax deposited by the oocyte to waterproof the egg is, therefore, quite different from the cuticular wax of this species; it cannot be looked upon as a form of cuticle laid down in the embryonic stages or suppressed into the developmental period. This means that the term *cuticle* cannot be applied to it.

If the primary waterproofing layer of wax had not been placed across the inner openings of the micropyles, it is certain that water could pass out through the micropyles and would be absorbed by the protein layer at the inner ends of the tubes. If the egg were in a dry atmosphere, the whole of the endochorion layers would be dry, so that the resistant endochorion protein layer would take up any water and distribute it throughout the whole of the chorion. Thus, the rate of water loss would be increased to a value considerably above that which might be expected if evaporation were to take place only from the bottoms of some fifteen narrow micropylar tubes. The necessity of having the primary wax layer over these minute openings is now apparent.

It must also be pointed out that shortly after the completion of the wax layer within the ovary, the egg is fertilized in the lower genital duct, and the spermatozoa must, therefore, be able to pass through this wax film.

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Separation of the 'blue' and 'green' mechanisms of foveal vision by measurements of increment thresholds

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The curve relating the smallest perceptible intensity of a blue test stimulus with the intensity of an orange conditioning field against which it is viewed shows a characteristic division into low- and high-intensity components, indicating the operation of two mechanisms of cone vision at the fovea. The justification for calling these 'blue' and 'green' mechanisms is taken from an earlier investigation (Stiles 1939). While most subjects show this division clearly, for some the low-intensity component is masked by the intrusion of rod vision. The correctness of this view is established by measurements made while the eye is recovering from an intense light adaptation. The individual variations of the sensitivities of the 'green' and 'blue' mechanisms in twenty subjects are assessed. Further evidence is obtained of an anomalously low threshold for the 'blue' mechanisms at very high conditioning fields of orange light.

INTRODUCTION

The operation of three receptor mechanisms in rod-free foveal vision can be demonstrated, and some of their properties, e.g. their spectral sensitivity curves, can be determined to a first approximation by measurements of the liminal brightness increment (l.b.i.) under suitable conditions (Stiles 1939).^{*} This conclusion rests mainly on measurements for one eye. A key feature of the results was the form of the curve relating the l.b.i. to the intensity of the conditioning stimulation for a test stimulus of short wave-length (below about 510 m μ) and a conditioning stimulation of long wave-length (above about 530 m μ). All such curves were found to show a 'change of law' enabling them to be represented as the resultant of two component curves, associated respectively with the 'green' and 'blue' cone

^{*} This paper is referred to as I throughout.

mechanisms, the former coinciding with the experimental curve at low-conditioning intensities, the latter at high. This association is justified by the way the component curves are displaced with respect to the co-ordinate axes when the wavelengths of test stimulus and conditioning stimulation are independently changed. From the observed displacements it appeared that the low intensity component curve was attributable to a mechanism having maximum sensitivity at a wavelength of 540 $m\mu$ approx., and the high intensity component curve to one having maximum sensitivity at 445 $m\mu$ approx.

In the present work the variation of the l.b.i. with conditioning intensity has been determined with a blue test stimulus and an orange conditioning stimulation for a group of twenty subjects. The results show a high-intensity 'blue' component in every case and in about half the cases the low-intensity 'green' component appears much as in investigation I. For some subjects, however, abnormally low values of the l.b.i. are obtained at low-conditioning intensities, suggesting that rod vision is playing a part. To test this point, the recovery of the eye after adaptation to an intense white light was determined by measurements of the l.b.i. for the blue test stimulus on a dark background. The recovery curves for the subjects in question show the characteristic two stages which Hecht (1937) and others have found to be associated with retinal areas where both rod and cone mechanisms are operative. With the aid of these recovery curves, the 'green' component curves can be determined approximately for all subjects, with one doubtful case. While confirming strongly the conclusion that the 'green' and 'blue' cone mechanisms can be separated by measurements of the l.b.i., the results reveal considerable variations among individuals both in the absolute and the relative sensitivities of the two mechanisms. In I it was observed that at a sufficiently high intensity of an orange or red conditioning stimulation further increase produced no corresponding increase in the l.b.i. of the 'blue' cone mechanism. From the present results it appears that this limited conditioning effect of orange or red light on the 'blue' mechanism occurs for most subjects.

By measurements of the l.b.i. with a different choice of colours for the test and conditioning stimuli, the 'green' and 'red' mechanisms can also be separated, although with more difficulty (I). Under the conditions of the present work, the 'red' mechanism is not in evidence and need not be referred to again.

2. EXPERIMENTAL DETAILS

The subject applied his eye to an artificial pupil and saw a circular patch of orange light of diameter 18° (the conditioning stimulation), containing at its centre four black fixation dots defining a square of 2° side (figure 1a). The test stimulus, a square patch of blue light of 1° side, was superposed on the orange background at the centre of the fixation square in flashes of 0.2 sec. once in every 1.4 sec. Its intensity was variable by the subject. The latter fixated carefully at the centre of the four black dots, and *after several minutes' adaptation to the*

orange field adjusted the test stimulus to be on the limit of visibility. Four or more settings were made. The intensity of the conditioning stimulation was then raised and the process repeated. For the special case of zero conditioning intensity, the dark fixation dots could not be seen and were replaced by four feeble points of orange light. A complete set of observations at 12 intensities from zero upwards occupied about 2 hr., including a preliminary period of about 20 min. dark adaptation.

The lay-out of the apparatus and its mode of operation are made clear by figure 1*b* and the following key:

- E* Artificial pupil of diameter 2.7 mm.
- S*₁ 500 W opal lamp run at a colour temperature of approximately 2700° K.
- X* Sheet of plain glass carrying four opaque spots of white paint which appeared as black dots when silhouetted against *S*₁. When *S*₁ was extinguished, the spots were faintly illuminated by a small lamp placed above the filters *F*_O, *F*_N (not shown).
- F*_O, *F*_N Chance's light orange glass and gelatine neutral filters (as required).
- C* Composite glass cube of 3 cm. side with half-reflecting diagonal surface.
- L*₁ Lens imaging the fixation points and diaphragm *D* at infinity: power to suit subject's refractive error, 4D for emmetropic eye.
- S*₂ Ribbon filament lamp run at colour temperature of approximately 2450° K.
- L*₂, *L*₃ Lenses of power 6.5 and 4D respectively giving a real image of *S*₂ on diaphragm *D*.
- F*_B, *F*_N Ilford's spectrum blue and gelatine neutral filter (if required).
- H* Rotating sector passing the light from *S*₂ for 0.2 sec. in every 1.4 sec.
- W*_x, *W*_y Opposed neutral wedges for varying continuously the intensity of the test stimulus.
- D* Diaphragm containing a square aperture covered by the image of *S*₂.

(The screening of the apparatus is not shown.)

The approximate relative energy distributions *E*_λ of the lights used for test and conditioning stimuli are plotted in figure 1*c*. Their colours approximate to those of monochromatic lights of wave-lengths 470 and 615 mμ respectively.

For tests on the recovery of the eye, a rectangular panel (5 × 12.5 cm.) of diffusing glass illuminated to a brightness of 770 candles/ft.² (white light) was mounted by the side of the main apparatus. The subject stared at this panel with both eyes from a distance of about 8 cm. for 10 min. At a given moment the panel illumination was extinguished and the subject quickly transferred his eye to the aperture *E* and signalled as soon as the test stimulus, set at a suitable intensity, became visible. The test stimulus intensity was then considerably reduced, by the experimenter, and again the subject signalled when he could just see it, and so on. In

the later stage of the recovery process the subject himself made settings to the liminal intensity. Throughout, lamp S_1 was kept extinguished so that the test stimulus appeared on a field of zero intensity.

The simpler apparatus and technique, compared with those used in I, were adopted mainly to make the tests less tiresome for the subject and to reduce the

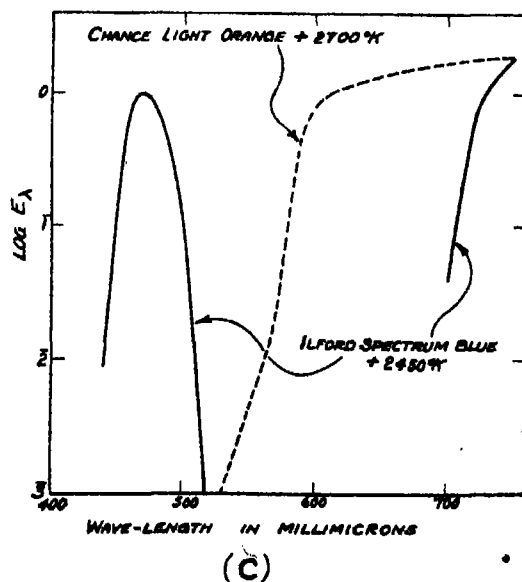
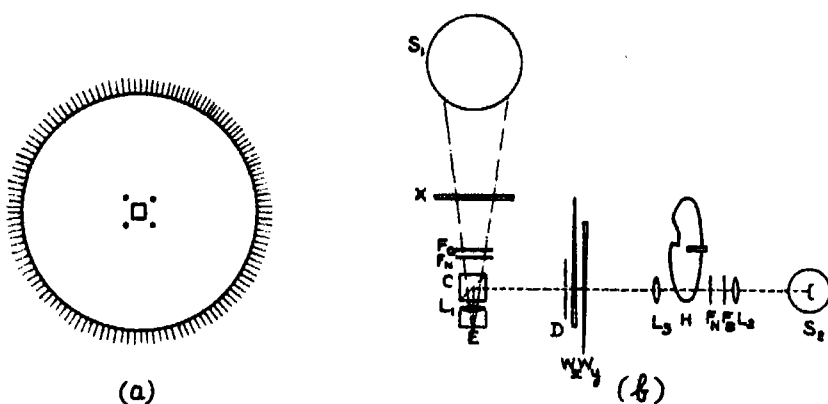


FIGURE 1

call on his time. The use of colour filters and non-Maxwellian view gives a 'cleaner' conditioning field than before, but, setting by the subject, the use of a material artificial pupil and the abandonment of the dental impression method for fixing the subject's head make for less satisfactory measurements. Details of the subjects are given in table 1.

TABLE 1

subject	sex	age	eye used in measurements	colour vision (normal unless otherwise indicated)
A	M.	43	left	—
B	F.	24	right	—
C	M.	53	right	—
D	M.	38	right	—
E	M.	37	left	—
F	M.	53	right	—
G	M.	38	right	—
H	M.	43	left	—
I	M.	33	left	—
J	F.	39	right	—
K	M.	34	right	deuteranomalous
L	M.	38	left	—
M	F.	18	right	—
N	M.	35	left	—
O	M.	50	right	—
P	M.	38	right	protanope
Q	F.	17	left	—
R	M.	46	right	—
S	F.	17	left	—
T	M.	23	right	—

3. CURVES SHOWING THE VARIATION OF THE LIMINAL BRIGHTNESS INCREMENT WITH THE CONDITIONING INTENSITY

The circle points of figure 2 show the observed variation of the l.b.i. with conditioning intensity for the writer's left eye. The cross-points are observations for this eye obtained in I* and refer to monochromatic test and conditioning stimuli of nearly the same colours as the present filtered lights. The two-component form is clearly shown by both sets of points, and the positions of the component curves along the axis of abscissae are in satisfactory agreement. Their positions along the ordinate axis would not be expected to agree precisely for three reasons:

- (i) the exposure time of the test stimulus has been increased (0.063–0.2 sec.);
- (ii) the effective wave-length of the test stimulus is slightly shorter (480–470 m μ approx.);
- (iii) the 'subject-setting' method gives slightly higher values for the l.b.i. than the 50 % probability method used in I (difference estimated at about 0.1 or 0.15 log unit).

The quantitative effects of (i) and (ii) can be estimated approximately, (i) from the Blondel-Rey law (1911), (ii) from the shapes of the spectral sensitivity curves of

* The l.b.i. U_λ and conditioning stimulation W_μ were then expressed in energy units but have been converted to photons by multiplying by $2.18 \times 10^8 V_{480}$ and $2.18 \times 10^8 V_{510}$ respectively, where V_λ is the C.I.E. relative visibility function. The photon is a unit of retinal stimulation: 1 photon is equivalent to 1 candle/m.² or 0.093 candle/ft.² seen through an artificial pupil of area 1 sq.mm.

the 'blue' and 'green' mechanisms. Including the effect of (iii), the circle points would be expected to fall below the cross-points by about 0.2 log unit ('green' component) and 0.3 log unit ('blue' component). Thus the agreement here is also very satisfactory.

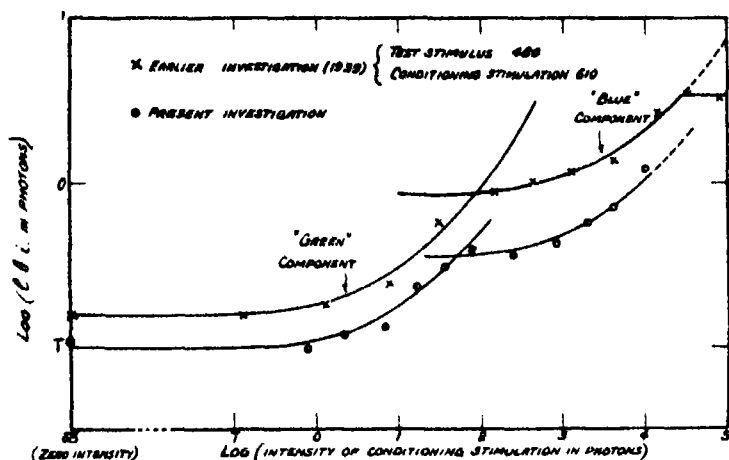


FIGURE 2. Observations for left eye of subject A (W.S.S.).

The (l.b.i./conditioning intensity) curves for the remaining nineteen subjects fall into three groups:

(a) those which can be represented as the resultant of two component curves (circle points of figure 3);*

(b) those like (a), except that at zero intensity the l.b.i. is abnormally low (circle points of figure 4);*

(c) those with abnormally low l.b.i. values at several of the low intensities (circle points of figure 5).*

By the resultant is to be understood a curve whose value at each intensity is equal, at least to a first approximation, to the lesser of the values of the two component curves.

For several subjects in each group the l.b.i. is anomalously low at one or two of the highest conditioning intensities. The discussion of this feature of the results is postponed to § 5.

The low values of the l.b.i. obtained at low intensities in groups (b) and (c) are almost certainly caused by the intrusion of rod vision. From the measurements of the recovery curve of the eye given below an approximate estimate of the l.b.i. of true cone vision at zero intensity can be made, and the values obtained are plotted as the square points in figures 4 and 5. Observations of the l.b.i. falling materially below the square-point value are to be attributed to rod response, and

* In figures 3-5 the scale of ordinates is correct for the top curve but must be shifted down 1 log unit for the second curve, 2 for the third, and so on.

if such observations are excluded the results for the subjects in groups (b) and (c) can be represented by two component curves as shown. In figure 5 the excluded points may be regarded as forming a third, *rod component* curve.

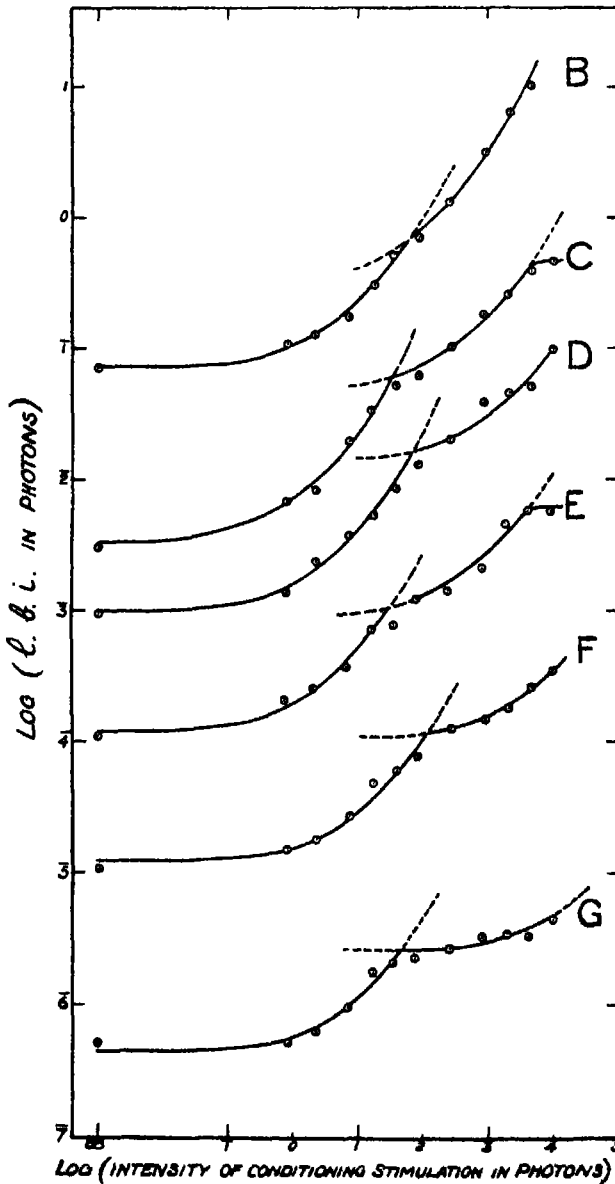


FIGURE 3. Observations for subjects in group (a).

All the cone component curves drawn in figures 2-5 are obtained from a curve of fixed shape which is displaced (without rotation) to different positions in the diagram to give for each subject the best fit with the experimental points in the high- or the low-intensity range. The basic curve of fixed shape is the one derived

in I for the writer's left eye ($-\log \xi(x)$ v. $\log x$, shown as curve 2 in figure 36 of I). On the whole this shape, when applied to the present measurements, is satisfactory for all subjects, but because of the comparatively short range of points on each

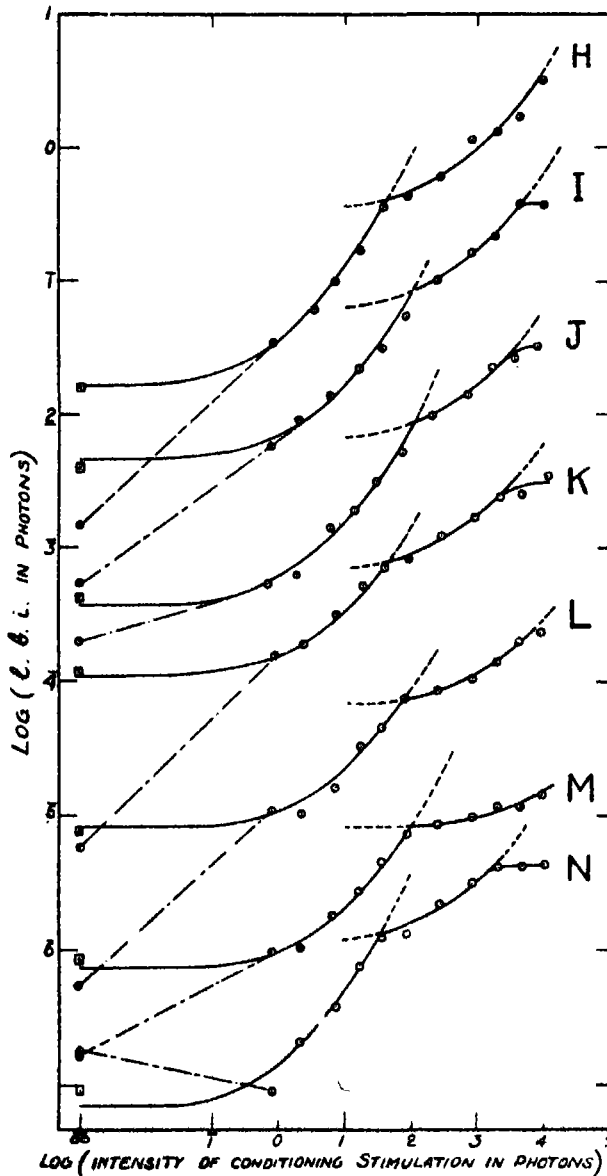


FIGURE 4. Observations for subjects in group (b).

component the test is not a critical one. However, the results are consistent with the view that the curve relating the l.b.i. of cone vision to the conditioning intensity is the resultant of two component curves of similar shape, the same for all

subjects, whose positions relative to the co-ordinate axes vary considerably from one subject to another. It is apparent from figures 3-5 that the changes of position are in general different for the two components, i.e. the *resultant curve* is not displaced bodily without change of shape.

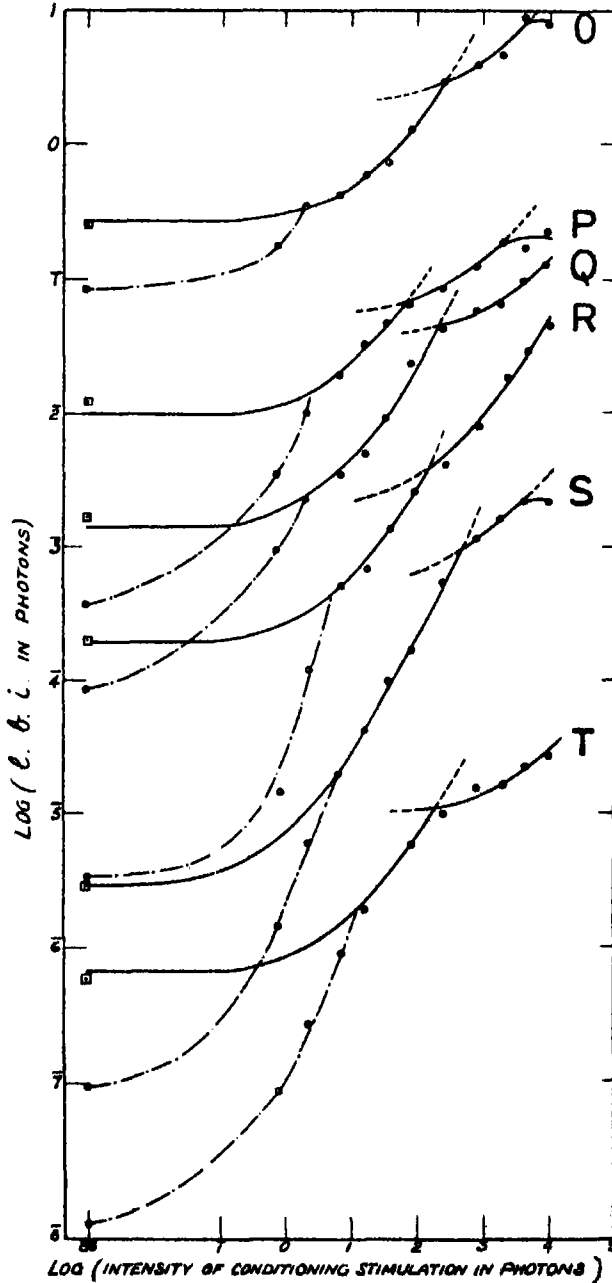


FIGURE 5. Observations for subjects in group (c).

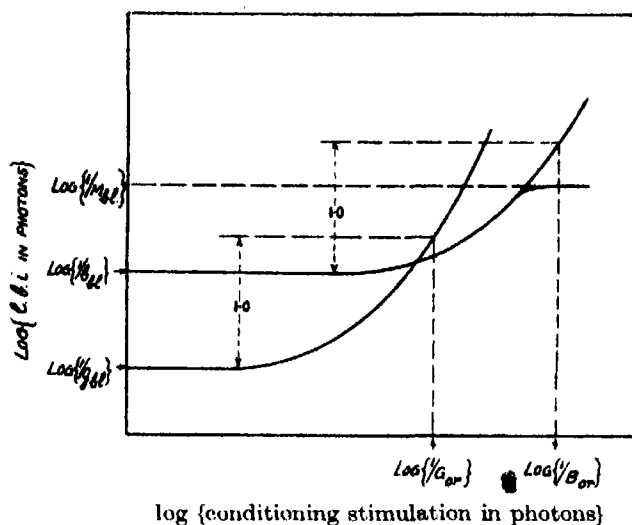


FIGURE 6. Derivation of the sensitivities of the mechanisms.

TABLE 2. SENSITIVITIES OF THE 'BLUE' AND 'GREEN' MECHANISMS FOR TWENTY SUBJECTS

(Expressed in reciprocal photons)

subject	log g_{bl}	log G_{or}	log (G_{or}/g_{bl})	log b_{bl}	log B_{or}	log (B_{or}/b_{bl})	log M_{bl}	log (b_{bl}/M_{bl})
A	1.06	3.73	4.67	0.41	5.08	5.67	—	—
B	1.12	2.16	3.04	0.44	4.95	4.51	—	—
C	1.36	2.59	3.23	0.29	4.30	4.01	1.32	0.97
D	1.03	2.45	3.42	1.84	5.84	4.00	—	—
E	0.88	2.40	3.52	0.08	4.30	4.22	1.25	0.83
F	0.92	2.00	3.08	1.92	5.16	5.24	—	—
G	1.34	2.00	4.66	0.61	5.67	5.06	—	—
H	1.78	2.86	3.08	0.45	4.08	5.63	—	—
I	1.36	2.37	3.01	0.16	5.97	5.81	1.40	0.76
J	1.45	2.42	4.97	0.15	5.86	5.71	1.50	0.65
K	0.93	2.18	3.25	0.13	5.93	5.80	1.50	0.63
L	1.11	2.15	3.04	0.13	5.41	5.28	—	—
M	1.20	2.14	4.94	0.06	5.70	5.64	—	—
N	1.09	2.74	3.65	1.99	4.40	4.41	1.36	0.63
O	0.56	3.82	3.26	1.64	5.65	4.01	1.05	0.59
P	1.04	2.01	4.97	0.21	5.93	5.72	1.68	0.53
Q	0.87	2.28	3.41	1.40	5.26	5.86	—	—
R	0.72	2.25	3.63	1.67	4.50	5.83	—	—
S	1.53	2.92	3.39	1.29	5.95	4.66	2.63	0.66
T	1.22	2.08	4.86	1.97	5.13	5.16	—	—

 g_{bl} = sensitivity of the 'green' mechanism to the blue test stimulus.

 b_{bl} = sensitivity of the 'blue' mechanism to the blue test stimulus.

 G_{or} = sensitivity of the 'green' mechanism to the orange conditioning stimulation.

 B_{or} = sensitivity of the 'blue' mechanism to the orange conditioning stimulation.

 M_{bl} = sensitivity of the 'blue' mechanism to the blue test stimulus when the conditioning stimulation has produced its maximum effect.

According to I, the high- and low-intensity components are respectively the l.b.i. curves which would be obtained if the 'blue' and 'green' cone mechanisms acted alone. Thus differences in position of the component curves are to be interpreted as differences in the sensitivities of these mechanisms. Sensitivity of a mechanism to the test stimulus is defined as the reciprocal of the l.b.i. at zero conditioning stimulation, and sensitivity to the conditioning stimulation as the reciprocal of the intensity of the latter required to raise the l.b.i. to ten times its value at zero intensity. The derivation of these sensitivities for the green and blue mechanisms from the positions of the corresponding component curves is made clear by figure 6 and the values obtained for the twenty subjects are set out in table 2.

4. RESULTS: RECOVERY CURVES

If the intrusion of rod vision is responsible for very low values of the l.b.i. at low intensities, we should not expect these low values to be reached rapidly after the eye has been exposed to a high brightness because of the known slow recovery (dark-adaptation) of the rod mechanism. The recovery curves for subjects Q and I belonging to groups (c) and (b) respectively, are reproduced on the left in figures 7 and 8, and indicate that a time of the order of 20 min. is required before the l.b.i. value previously observed at zero intensity is reached. Moreover, the curves show an initial cone phase and a later rod phase and the final l.b.i. of true cone vision is clearly defined. These final cone values are transferred as the square points in the (l.b.i./conditioning intensity) curves shown on the right in the same figures. The recovery curves for the extreme types in group (a) (subjects B and G) show the more rapid single phase recovery curve appropriate to pure cone vision (figure 9).

The recovery curves for the remaining subjects were in general similar to those just discussed although in one or two cases (particularly for subject S), the cone to rod transition is less marked and the uncertainty in fixing the true cone l.b.i. at zero intensity is greater.

There are three reasons which could account for the intrusion of rod vision for some subjects:

- (i) their foveas may contain rods or, at the least, the rod-free area may be smaller than the 1° square test stimulus;
- (ii) they may have failed to maintain strict foveal fixation;
- (iii) foveal fixation as normally understood may have been maintained but their rod-free areas may be displaced.

As regards (i), Abney & Watson (1916) found that three out of eight subjects showed characteristic rod properties at the fovea (test light, diam. 34 min.) and were emphatic that fixation errors were not responsible. In the present measurements, cause (ii) was certainly operating for some subjects. With the blue test stimulus used the ratio of rod to cone sensitivities reaches its highest value and every one had difficulty in fixating at zero conditioning stimulation. Subjects K

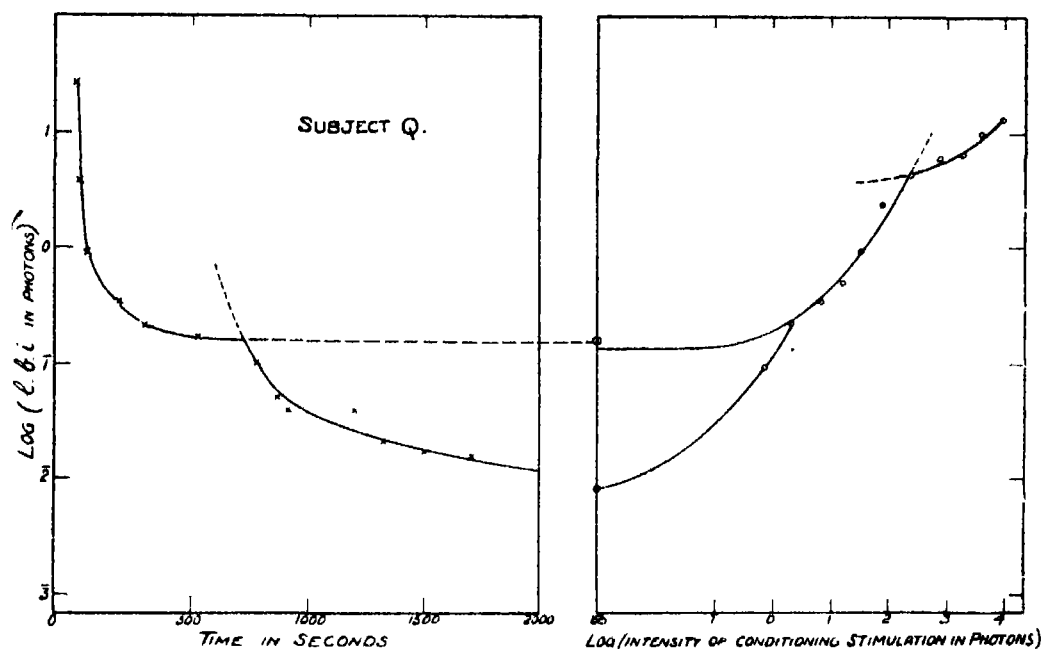


FIGURE 7. Recovery curve and (l.b.i./intensity level) curve.

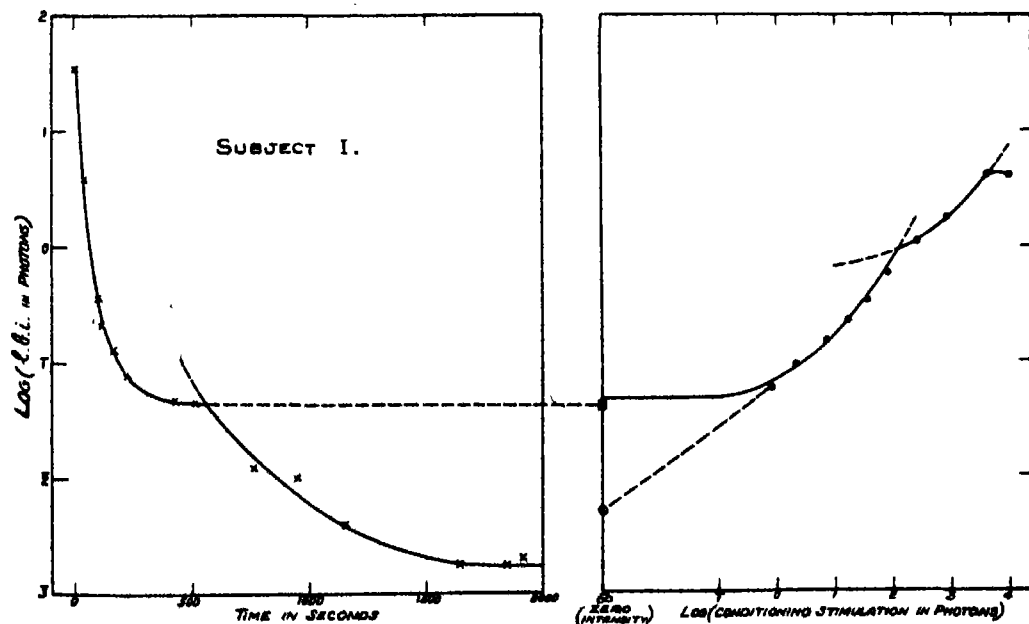


FIGURE 8. Recovery curve and (l.b.i./intensity level) curve.

and O gave single phase recovery curves and did not repeat the abnormally low values of the l.b.i. at low intensities, obtained in the original (l.b.i./conditioning intensity) runs. On the other hand, one subject (N) gave a well-defined two-phase recovery curve, reaching lower values of the l.b.i. in the rod phase than had been obtained previously under steady conditions at zero intensity. These differences are attributable to varying success in maintaining fixation on different occasions.

Cause (iii) is certainly operative for one subject T for whose retina, it is known from other work, a rod-free response at low intensities can be obtained if the direction of fixation is about $\frac{1}{2}^{\circ}$ to the left of the test stimulus.

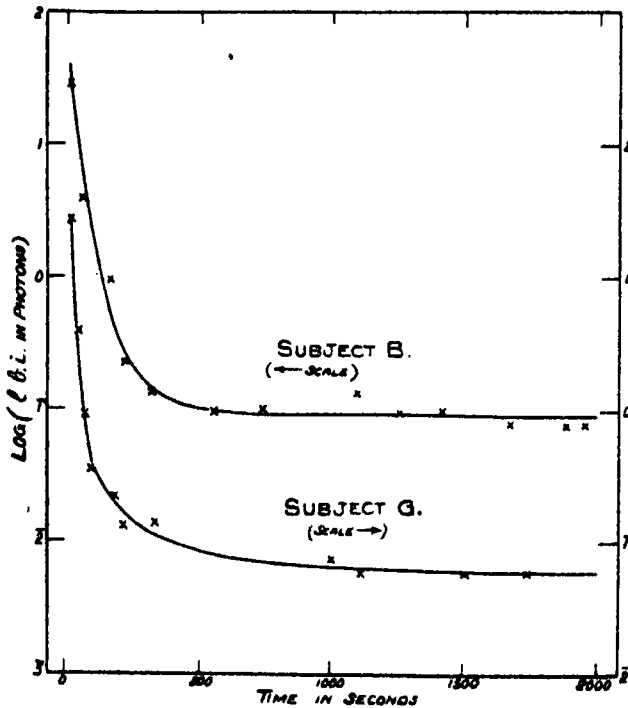


FIGURE 9. Recovery curves.

5. LIMITED CONDITIONING EFFECT OF RED LIGHT ON THE 'BLUE' CONE MECHANISM

In figure 2, upper curve, the l.b.i. for the highest conditioning intensity is about 0.3 log unit below the value expected from the 'blue' component curve. This is not just a bad observation but an example of an effect consistently observed in I, that as the intensity of an orange or red conditioning stimulation was increased it raised the l.b.i. of the 'blue' mechanism in the normal way to a certain value and thereafter produced no further increase. For green or blue conditioning stimulations this did not occur. The intensity available in the present apparatus was insufficient to reach the range of constant l.b.i. for the writer's eye (see lower curve of figure 2),

but for several other subjects it appears to have been reached. Most of the observed l.b.i. values belonging to the high-intensity range in figures 2-5 differ from the 'blue' component curve as drawn by less than 0.1 log unit. Suppose the results of a particular subject are accepted as showing a limited conditioning effect if the observed l.b.i. at the highest intensity lies below the 'blue' component curve as drawn by 0.2 log unit or more. On this basis nine subjects show it, the effect being most marked for subject N. From the results for these cases, estimates have been made of M_{bl} , the reciprocal of the upper limiting value of the l.b.i. of the 'blue' mechanism (see figure 6). The log of this quantity and of the ratio b_{bl}/M_{bl} are given in table 2. Log (b_{bl}/M_{bl}) takes values ranging from 0.53 to 0.97 with an average of 0.69 compared with the value 0.63 obtained in I as the mean from many curves for the writer's left eye.

It appears therefore that limited conditioning sets in when the conditioning stimulation is sufficient to raise the log (l.b.i.) of the 'blue' mechanism by about 0.7 log unit. From the shape of the standard component curve it is readily determined that if L and N are the conditioning intensities required to raise the l.b.i. of the 'blue' mechanism by 1.0 and 0.7 respectively, then $\log N = \log L - 0.45$. We should not expect to observe limited conditioning if $\log N$ exceeds 4.00, the log of the maximum conditioning intensity used in the measurements. But by definition $\log B_{or} = \log (1/L) = -\log L$, so that $\log N > 4.00$ is equivalent to $\log B_{or} < \bar{5}.55$. From the values of log B_{or} given in table 2, it is seen that none of the subjects for whom $\log B_{or} < \bar{5}.55$ shows limited conditioning in the present tests, although two (A and K) are known from other work to show it at higher intensities. Of the thirteen subjects for whom $\log B_{or} > \bar{5}.55$, nine show the effect. In sum, therefore, $9 + 2 = 11$ subjects out of $13 + 2 = 15$ are known to show limited conditioning (about 3 in 4), while the remaining five subjects would not be expected to show it in the present measurements.

The anomalously low l.b.i. values at high intensities are regarded as a special property of the 'blue' mechanism and not as the initial part of the component curve of an additional mechanism. The reasons for this view are given in I.

6. INDIVIDUAL VARIATIONS IN THE SENSITIVITIES OF THE 'BLUE' AND 'GREEN' MECHANISMS

In figure 10 the sensitivity to the test stimulus is plotted against the sensitivity to the conditioning stimulation for each subject and for the two mechanisms. The total range of variation and the standard deviations of the log sensitivities have the following values:

	total range	standard deviation
$\log \rho_{bl}$	1.2	0.28
$\log G_{or}$	1.2	0.31
$\log b_{bl}$	1.3	0.34
$\log B_{or}$	2.3	0.60

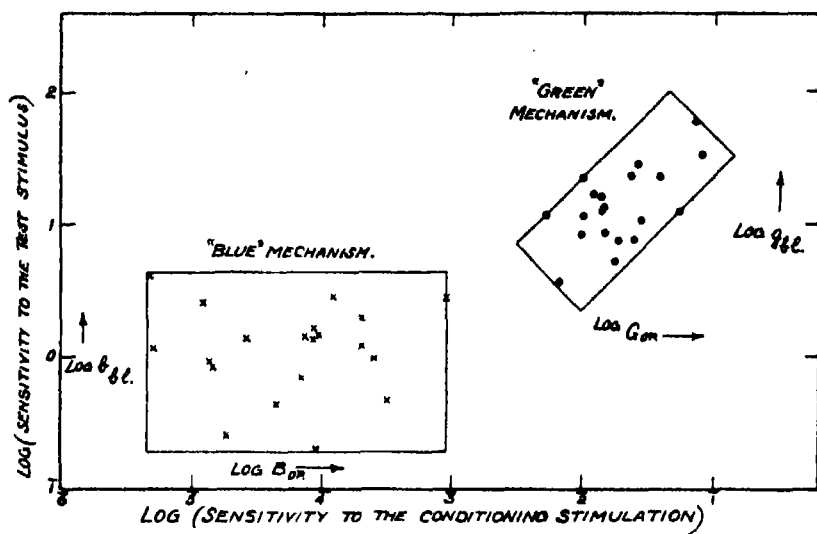


FIGURE 10

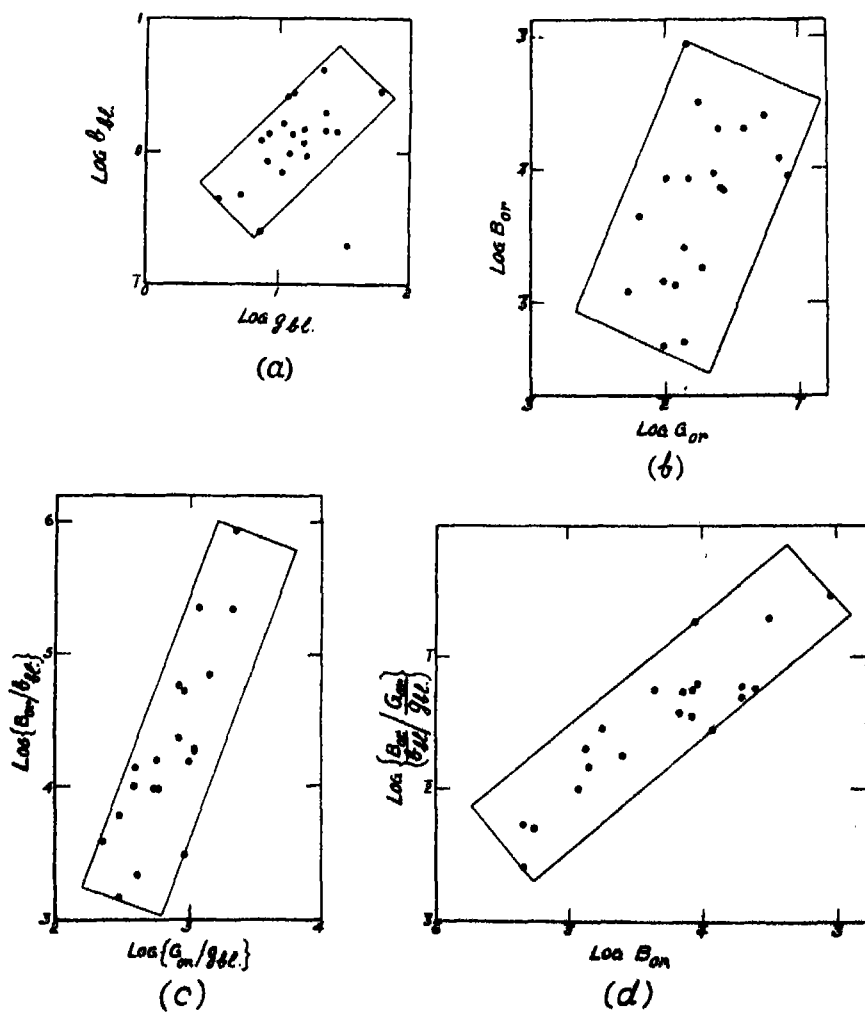


FIGURE 11

The spread arises in part from uncertainty in fitting the component curves to the observations, in part from a true individual variation which appears to be greatest for B_{or} . For the 'green' mechanism there is a weak correlation of the two sensitivities $\log g_{bl}$ and $\log G_{or}$ but for the 'blue' mechanism there is no correlation.

The following points are illustrated in figure 11:

(i) $\log b_{bl}$ v. $\log g_{bl}$. The sensitivities to the test stimulus tend to increase together and proportionally for the two mechanisms but subject S appears to be exceptional in this respect (figure 11a).

(ii) $\log B_{or}$ v. $\log G_{or}$. The sensitivities to the conditioning stimulation show a slight tendency to increase together but not proportionally (figure 11b).

(iii) $\log g_{bl}$ v. $\log B_{or}$ and $\log b_{bl}$ v. $\log G_{or}$. No correlation (not reproduced).

(iv) $\log (B_{or}/b_{bl})$ v. $\log (G_{or}/g_{bl})$. These log ratios tend to increase together but not proportionally (figure 11c).

(v) $\log \left\{ \frac{B_{or}}{b_{bl}} \middle/ \frac{G_{or}}{g_{bl}} \right\}$ tends to increase proportionally with $\log B_{or}$ (figure 11d) but shows no correlation with $\log b_{bl}$, $\log G_{or}$ or $\log g_{bl}$.

The quantity $\log \left\{ \frac{B_{or}}{b_{bl}} \middle/ \frac{G_{or}}{g_{bl}} \right\}$ is of some interest as for monochromatic test and conditioning stimuli it would remain unchanged if the subject made all his observations through a colour filter of arbitrary spectral transmission and this fact was ignored in working out the results so that the filter was treated as part of the eye. If such a colour filter had transmissions t_{or} and t_{bl} for the orange and blue lights, it is clear that the observed sensitivities would become $B_{or}t_{or}$, $G_{or}t_{or}$, $b_{bl}t_{bl}$, $g_{bl}t_{bl}$, respectively, but we should have

$$\log \left\{ \frac{B_{or}t_{or}}{b_{bl}t_{bl}} \middle/ \frac{G_{or}t_{or}}{g_{bl}t_{bl}} \right\} = \log \left\{ \frac{B_{or}}{b_{bl}} \middle/ \frac{G_{or}}{g_{bl}} \right\}.$$

The effective widths of the spectrum bands corresponding to the present non-monochromatic stimuli are small and it may fairly be concluded from the wide variation of

$$\log \left\{ \frac{B_{or}}{b_{bl}} \middle/ \frac{G_{or}}{g_{bl}} \right\}$$

that individuals differ in a way which cannot be assimilated to selective absorption by a pigment layer.

The outstanding feature in the above correlations, which are not of course all independent, is the large and dominating variation of $\log B_{or}$, the sensitivity of the 'blue' mechanism to orange light.

7. CONCLUSIONS

1. Measurements of the liminal brightness increment show for all subjects the operation of two cone mechanisms ('green' and 'blue') in foveal vision.

2. For some subjects, rod vision intrudes at low intensities, but this difficulty can be dealt with by determining the cone l.b.i. before the rods have had time to recover from intense light adaptation.

3. Individual variations are most marked in the sensitivity of the 'blue' cone mechanism to orange light.

4. Most subjects give evidence of a limited conditioning effect of orange light on the 'blue' cone mechanism.

The work described above has been carried out as part of the research programme of the National Physical Laboratory and this paper is published by permission of the Director.

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Experiments on the inactivation of bacteriophage by radiations, and their bearing on the nature of bacteriophage

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Three bacteriophages (S-13, C-36 and Staph-K) were irradiated by γ -rays, X-rays and α -rays. The survival curves were exponential, and the effect of a given dose was independent of the exposure time. For any given phage the inactivation doses of the three radiations increased in the order γ -rays, X-rays, α -rays, while for any given radiation the inactivation doses of the three phages diminished in the order S-13, C-36, Staph-K, which is the order of increasing size.

These observations lead to the conclusion that a single ionization suffices to inactivate a phage particle. In the case of the smallest phage investigated (S-13) this ionization is effective wherever in the particle it is produced, and reasons are given for concluding from this fact that S-13 is a macromolecular type of virus analogous to the crystallizable plant viruses.

In the case of the larger phages (C-36 and Staph-K), while a single ionization can inactivate a phage particle it is not sufficient for it to be produced anywhere in the phage particle; to be effective it must be produced in a more restricted region. It is suggested that this radiosensitive region constitutes the genetical material of the phage, and on the basis of this differentiation into genetic and non-genetic material these phages are regarded as primitive single-celled organisms rather than macromolecules.

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INTRODUCTION

Studies of the inactivation of viruses by ionizing radiations (i.e. X-rays and the radiations from radioactive substances) are of interest from two points of view. On the one hand, virus inactivation is one of the simplest biological actions of radiation, and, therefore, makes a good starting-point for a study of this complicated subject. On the other hand, one may hope to use radiation as a tool in the investigation of the nature of the viruses themselves, encouraged by the success which has attended its use by geneticists in the study of mutation.

Included among the viruses there are at the one extreme the crystallizable plant viruses, the individual particles of which may reasonably be regarded as macromolecules, and at the other extreme larger bodies which some workers have regarded as akin to single-celled organisms. We shall in this paper distinguish between the two types of virus as *macromolecular virus* and *organism-type virus*, being of the opinion that the term *organism* is better confined to the second type. The distinction is most clearly expressed in genetical terms. The suggestion has long been current (Muller 1922) that the smallest viruses are of the nature of single, isolated genes. In higher cells there is a differentiation between the genetic and the non-genetic material of the cell, the former component comprising only a small fraction of the total bulk of the cell. If the larger viruses are single-celled organisms, a similar differentiation may be expected in them.

We have suggested (Lea & Salaman 1942) that the study of the inactivation of viruses by radiation can be used as a method to distinguish between the two types of virus. The basis of the method is as follows. The internal evidence of the radiation experiments indicates that, with all types of viruses, inactivation of a virus particle is due to a single ionization. (The arguments involved are set out at length in Lea (1946) and are referred to later in the present paper.) The usual effect of the ionization of an atom is the decomposition of the molecule of which it is a part. This has been known for a long time from studies of the decomposition of simple inorganic compounds by radiations (Lind 1928), and has been shown to be true also for the decomposition of protein molecules, as a result of studies of the inactivation of enzymes by X-rays (Lea, Smith, Holmes & Markham 1944). We conclude therefore that when a virus particle is inactivated (by this term we mean rendered unable to multiply and produce its characteristic symptoms in the host), inactivation is due to chemical change in a single molecule.

In the case of a macromolecular virus, this means that an ionization *anywhere* in the virus will cause inactivation, and this expectation has been shown to be at any rate approximately true by studies on plant viruses (Gowen 1940; Lea 1940b; Lea & Smith 1942) and on bacteriophages (Exner & Zaytzeff-Jern 1941; Luria & Exner 1941; Exner & Luria 1941; Wollman, Holweck & Luria 1940).

In the case of an organism in which there is differentiation into genetic and cytoplasmic components, it seems improbable that the content of any cytoplasmic component is so exactly balanced that the destruction of a single molecule of it

leads to death of the organism. On the other hand, there is nothing improbable in the notion that chemical change in a gene molecule should have a lethal effect. The geneticist is familiar with mutations in *Drosophila* which (when not concealed by the presence of an unchanged homologous gene) are lethal to the cells containing them, and some of these mutations are due to internal changes in single genes. Thus, if in a virus inactivation by a single ionization is observed, it may be concluded that this is due to the production of an ionization, not anywhere in the virus, but in its genetic material.

The determination of the dose of radiation required to inactivate a virus makes it possible to calculate the volume of the sensitive (genetic) material in which a single ionization can cause inactivation. When dealing with a macromolecular virus one would expect to obtain a volume approximately equal to the whole volume of the virus, but when dealing with the organism-type of virus one would expect to obtain a volume smaller than the whole volume of the virus. As already mentioned, this test applied to the crystallizable plant viruses confirms that they are macromolecules. Applied to vaccinia (Lea & Salaman 1942) it appeared that only a small fraction of the whole virus (about $\frac{1}{2}$ %) consisted of genetically important radiosensitive material, and we concluded that vaccinia was an organism and not a macromolecule.

The distinction between macromolecule and organism-type of virus can be made if data concerning the inactivation of the virus by any one type of radiation is available. Given data for several radiations of differing ion-density, then, in the case of the organism-type of virus, it is possible to make some deductions regarding the distribution of the genetic material in the cell, and in particular to make a rough estimate of the number of genes. The method is the same as one which has been proposed for determining the number of genes in the *X*-chromosome of *Drosophila* (Lea 1940a), where it gives a result in fair accord with other data. Owing to the sensitivity of the calculation of the number of genes to errors in the experimental data or to inadequacies in the method of calculation, the method is not an accurate one, and applied to viruses its principal use is to determine whether or not there is a multiplicity of genes. Applying it to vaccinia (Lea & Salaman 1942) we came to the conclusion that in this virus there is certainly a multiplicity of genes. We also stated that the radiosensitive material was contained in a region which presented an area of about one-quarter that presented by the whole virus particle. Since making these deductions, electron-micrographs (Green, Anderson & Smadel 1942, confirmed subsequently by Salaman & Preston, unpublished) have shown that the vaccinia particle contains internal structures, possibly chromosomes in which the genes are located, consistent in size with our deductions from the radiation data.

In the experiments reported in the present paper we have studied the inactivation by radiation of three bacteriophages of different sizes. The conclusion we have reached is that the smallest phage studied (S-13) is certainly of the macromolecular type, since the size of the sensitive region determined from the radiation experi-

ments is identical with the size of the phage particle itself. In the case of the larger phages (C-36 and Staph-K) the volume of the radiosensitive material is rather less than the total volume of the phage particle, indicating some differentiation into genetic and cytoplasmic material. The genetic material occupies a larger fraction of the total volume than in the case of vaccinia. There is a multiplicity of genes, but the number is small. Altogether, the indications are that these two phages are organism-type viruses, but less complex than vaccinia.

EXPERIMENTAL METHODS

The radiological methods were very similar to those used in the earlier paper (Lea & Salaman 1942) and will be only briefly described. Cover-slips were coated with gelatin, and a drop of a phage suspension was placed on each cover-slip and dried at 37°C; thus a thin film of phage was obtained which could be penetrated by radiations of low penetrating power. Three types of radiation were employed, namely, the γ -rays of radium, X-rays of wave-length 1.5 Å, and α -rays, the latter emitted by a source which in most of the experiments consisted of polonium deposited on a disk of silver, and in some of the experiments of the active deposit from radium emanation deposited on the flat end of a brass rod. The last-mentioned source decayed gradually after removal of the brass rod from the radium emanation. Its α -ray activity was measured before and after the exposure of the phage, and the dose of α -radiation received by the phage calculated from these measurements, making use of standard tables of the decay of active deposit (Curie 1935). The exposure of the phage did not commence until at least 15 min. after the removal of the brass rod from the radium emanation. Under these conditions the α -rays were almost entirely those of Ra C'.

After irradiation with the desired dose the cover-slip was dropped into a tube of water at 37°C, when the gelatin dissolved and the phage became suspended in the water. Suitable dilutions were then plated.

The phages were obtained from the National Institute for Medical Research. They were phage Staph-K (sensitive organism Staph S3K), phage C-36 (sensitive organism *Bact. coli*), and phage S-13 (sensitive organism *Bact. dysenteriae* Y6R). Phage suspensions were prepared by the usual technique of adding a drop of phage to a broth culture of the sensitive organism growing at 37°C, incubating until clearing took place, and then heating for an hour at a temperature (58°C) lethal to the bacteria but not to the phage, and finally spinning off the bacterial debris. In some cases the phage preparations were filtered through Berkefeld or Gradocol filters instead of being heated. This procedure was always used with phage S-13 which is inactivated if heated to 58°C for an hour. The stock suspensions obtained in this way usually gave one to ten plaques per ml. at a dilution of one to a million.

For the estimation of the phage suspensions the usual plaque-counting technique was employed. A simple agar-peptone-Lemco-NaCl medium containing 1 % of Evans's peptone was found suitable, 2 % agar being used for the S-13 phage, and

1.2 % agar for the other phages, which gave inconveniently small plaques on more concentrated agar. A drop of a suspension of the sensitive organism containing about ten million bacteria was placed on a plate of the medium, together with a measured drop of a suitable dilution of the phage suspension to be tested, and spread with a glass spreader. Plaques were counted after about 18 hr. incubation. With phages C-36 and Staph-K a suitable density of plaques was 50–200 on a 9 cm. diameter Petri dish. With phage S-13, which gave larger plaques, 20–50 was preferred. With these densities we found plaque count directly proportional to phage inoculum, and no calibration curve relating plaque count to phage inoculum such as was found necessary by Dreyer & Campbell-Renton (1933), who used very high densities of inoculation, was required.

RESULTS

The results of the main series of radiation experiments are exhibited in figure 1. For each of the three phages and for each of the three radiations a curve is given showing how the phage activity diminishes with increase of the dose of radiation. Radiation doses are measured in roentgens, and the phage activity is represented by the natural logarithm (to base $e = 2.718$) of the ratio of the plaque count of the irradiated sample to the plaque count of a control identically treated except for the absence of radiation. Plotted in this way it is seen that the experimental points fall satisfactorily on straight lines. Each point is an average of from two to five separate determinations, and a total of about 45,000 plaques were counted in the experiments summarized in figure 1.

The *inactivation dose* is conventionally taken to be the dose which reduces the natural logarithm of the surviving activity to $\bar{1}$, i.e. which reduces the ratio of experimental to control plaque count to a fraction 0.37. In table 1 we give the inactivation doses of the three phages for each of the three radiations.

TABLE 1. INACTIVATION DOSES OF THREE PHAGES FOR DIFFERENT RADIATIONS

radiation	Doses are in millions of roentgens				
	γ -rays	X-rays (1.5 A)	Ra C' α -rays		polonium α -rays 4 eMV
			7 eMV	4 eMV	
phage S-13	0.58 \pm 0.03	0.99 \pm 0.05	—	—	3.50 \pm 0.42
phage C-36	0.21 \pm 0.01	0.43 \pm 0.03	—	—	0.94 \pm 0.03
phage Staph-K	0.079 \pm 0.004	0.109 \pm 0.006	0.21 \pm 0.02	0.59 \pm 0.08	0.45 \pm 0.04

Experiments were made in the case of two of the phages, C-36 and Staph-K, in which the effect of a dose of X-rays given over a long time at low intensity was compared with the effect of an equal dose concentrated in a short time at high intensity. The results of these experiments are given in table 2. For a twenty-fold change of intensity a change of only 10–20 % in the inactivation dose was obtained, which was interpreted to mean that, within the experimental error, the effect of a given dose of radiation does not vary with its intensity.

TABLE 2. INACTIVATION DOSES AT DIFFERENT INTENSITIES OF RADIATION

phage	intensity r./min.	exposure min.	dose r.	surviving fraction	inferred inactivation dose r.
Staph-K	1.34×10^4	38	0.510×10^6	0.00425	0.0935×10^6
	4.72×10^3	1014	0.479×10^6	0.00298	0.0822×10^6
C-36	1.54×10^4	60	0.924×10^6	0.162	0.508×10^6
	8.13×10^3	1005	0.817×10^6	0.140	0.417×10^6

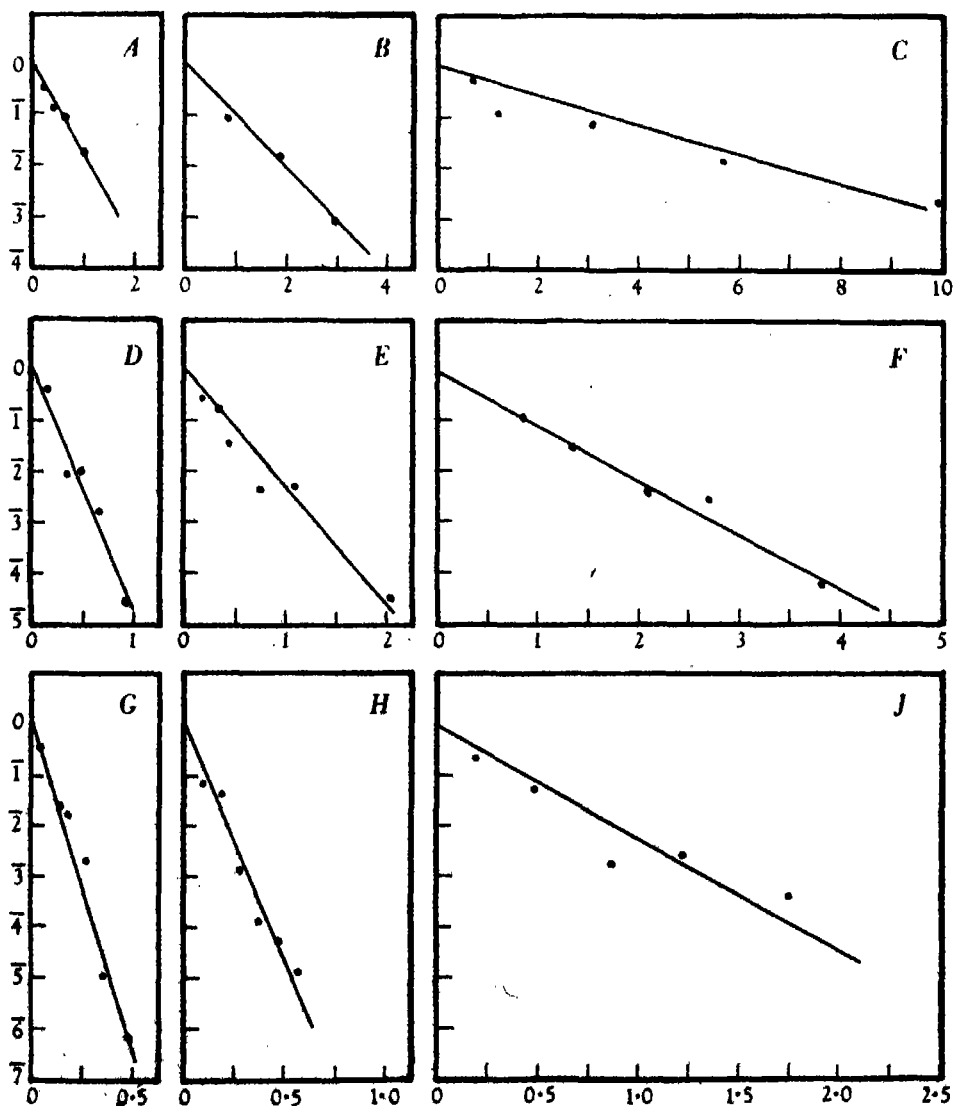


FIGURE 1. The inactivation of bacteriophages by γ -rays, X-rays and α -rays. Abscissae: dose in millions of roentgens. Ordinates: natural logarithm of fraction of activity surviving.

A γ -rays } on phage S.13. D γ -rays } on phage C-36. G γ -rays } on phage Staph-K.
 B X-rays } E X-rays } H X-rays }
 C α -rays } F α -rays } J α -rays }

In the case of phage Staph-K, in addition to the experiments using polonium as the source of α -particles, some experiments were made using Ra C' α -particles obtained from the active deposit obtained on the end of a brass rod exposed to radium emanation. Two series of experiments were made, one in which the α -particle source was unscreened, the average energy of the α -particles traversing the film of virus being estimated as 7 eMV, and the second series in which the α -particles were reduced in energy by the interposition of an aluminium foil of superficial mass 4.74 mg./cm.^2 between the source and the phage, the average energy of the α -particles traversing the film in this case being estimated as 4 eMV, approximately the same as when polonium (unscreened) was used as the source of α -particles. In figure 2 the survival curves obtained in this experiment are plotted in a manner similar to that explained in connexion with figure 1. The inactivation doses read off from these curves are included in table 1.

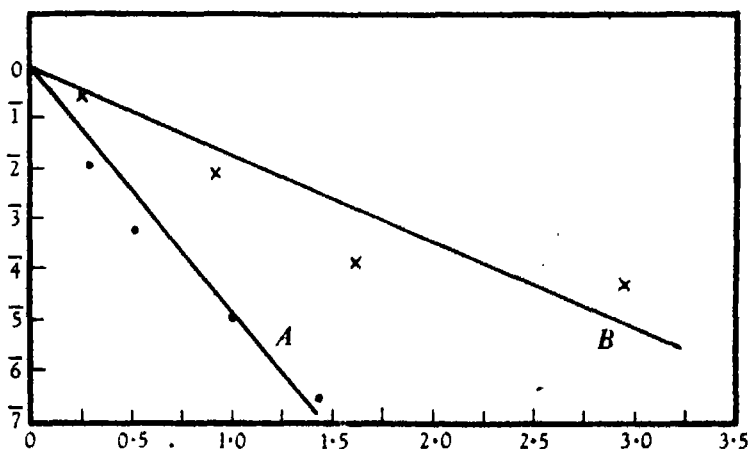


FIGURE 2. The inactivation of phage Staph-K by Ra C' α -particles. Abscissae: dose in millions of roentgens. Ordinates: natural logarithm of fraction of activity surviving. A, 7 eMV α -rays; B, 4 eMV α -rays.

INTERPRETATION OF THE RESULTS

The interpretation of the radiation experiments requires a knowledge of the sizes of the three bacteriophages employed. The data available have been summarized by Elford (1938). The sizes relevant are those of the dried particles, and will be somewhat smaller than the sizes determined in aqueous solution when the particles are hydrated. The results of filtration experiments (Elford & Andrewes 1932) need to be reinterpreted by making use of the revised relation between particle size and average pore diameter proposed by Markham, Smith & Lea (1942). The following appear to be the best estimates which can at present be made of the diameters of the unhydrated phage particles, assumed spherical: S-13 $16 \text{ m}\mu$, C-36 $42 \text{ m}\mu$, Staph-K $64 \text{ m}\mu$.

Qualitatively, the results described in the last section can be summarized as follows:

(a) The survival curves are exponential (i.e. when the logarithm of the fraction surviving is plotted against dose of radiation, the points lie approximately on straight lines). This result is in agreement with previous experiments on the irradiation of bacteriophages (e.g. Wollman & Lacassagne 1940; Wollman *et al.* 1940; Luria & Exner 1941). It is further in agreement with experiments on the irradiation of plant viruses (e.g. Lea & Smith 1942) and animal viruses (e.g. Syverton, Berry & Warren 1941; Lea & Salaman 1942).

(b) The effect of a given dose is independent of whether it is given in a short time at high intensity or is spread over a long time at low intensity. This result is in agreement with a previous experiment on the irradiation of a bacteriophage (Latarjet 1942), and is further in agreement with an experiment on an animal virus (vaccinia, Lea & Salaman 1942).

(c) With any one of the three phages, the inactivation dose increases with increasing ion-density of the radiation, i.e. in the order γ -rays, X-rays, α -rays. With phage Staph-K, where two α -ray energies were used, the inactivation dose increases with diminishing α -ray energy, which again is in the direction of increasing ion-density. This is in agreement with an experiment of Wollman *et al.* (1940), who irradiated phage C-16 and found that the inactivation dose was greater for α -rays than for X-rays. It is further in agreement with experiments on the inactivation of plant viruses (Lea & Smith 1942) and of an animal virus (Lea & Salaman 1942).

(d) With any one of the three radiations, the inactivation doses diminish in the order of increasing size of the phage particles. This is in agreement with the experiments of Wollman & Lacassagne (1940), who irradiated a number of bacteriophages with X-rays.

Results (a), (b) and (c) indicate that the inactivation of a phage particle is accomplished by a single ionization. The reasoning involved has been used in previous papers (Lea & Smith 1942; Lea & Salaman 1942). It may be found set out at length elsewhere (Lea 1946), and will not be discussed further here.

Having established that inactivation of a phage particle is accomplished by a single ionization, the question arises whether the ionization is equally effective if produced anywhere in the phage particle or whether it must be produced in some restricted region or 'target' of the phage particle. The method of deciding this question is to suppose that to inactivate the phage particle it is necessary to produce an ionization in a volume $v \mu^3$, to calculate the volume v from the measured inactivation dose, and to compare it with the known size of the particle. The calculation of the volume v from the inactivation dose is in principle simple. To inactivate we must produce ionizations at the rate of one per volume v , i.e. at the rate $(1/v)$ ionizations per μ^3 , and this, multiplied by the appropriate factor (about 0.5) to convert ionizations per μ^3 to roentgens, is the inactivation dose corresponding to a target volume v . In practice the calculation is more complicated, owing to the

non-random spatial distribution of the ionization in irradiated material; calibration curves are, however, available (Lea 1946, improving upon calibration curves published by Lea & Smith 1942), from which the diameter of the target (presumed spherical) may be read off for any given inactivation dose obtained with any given radiation.

In the case of phage S-13 the target sizes calculated from the inactivation doses with each of the three radiations employed agree closely, and agree also with the size of the unhydrated particle deduced from filtration and centrifugation experiments, as shown in table 3. With this phage therefore the ionization of any atom in the phage particle leads to the inactivation of the phage, and in accordance with the principles discussed in the Introduction, we conclude that phage S-13 belongs to the macromolecular type of virus.

TABLE 3. PHAGE S-13. UNHYDRATED PARTICLE DIAMETER $\sim 16\text{m}\mu$

radiation	γ -rays	X-rays	α -rays (4 eMV)
target diameter ($\text{m}\mu$)	15.5	15.9	16.3

With the phages C-36 and Staph-K the results are not so simple. As indicated in tables 4 and 5, the target diameters for all radiations are smaller than the phage particle diameters, and further, different radiations give different target diameters, greater for α -rays than for γ -rays. The first of these discrepancies indicates that not the whole of the virus particle is radiosensitive; there are many atoms which can be ionized without inactivation resulting. In accordance with the principles discussed in the introduction, we conclude that phages C-36 and Staph-K, in contrast to S-13, are organism-type viruses and not macromolecules.

TABLE 4. PHAGE C-36. UNHYDRATED PARTICLE DIAMETER $\sim 42\text{m}\mu$

radiation	γ -rays	X-rays	α -rays (4 eMV)
target diameter ($\text{m}\mu$)	21.5	22.3	33

TABLE 5. PHAGE Staph-K. UNHYDRATED PARTICLE DIAMETER $\sim 64\text{m}\mu$

radiation	γ -rays	X-rays	α -rays		
			7 eMV Ra C'	4 eMV Ra C'	4 eMV Po
target diameter ($\text{m}\mu$)	31	40	58	43	50

The fact that the estimates of target diameter given by the different radiations are not in agreement means that the radiosensitive part of the phage particle cannot be regarded as a single spherical region; it must be markedly aspherical in shape, or else the radiosensitive part of the phage particle must be distributed in a number of parts (cp. Lea & Salaman 1942; also Lea 1946). If we consider the second possibility as being, by analogy with other organisms, the more likely, and interpret inactivation of a virus by radiation as lethal mutation, then radiosensitive material distributed as a number of units can be interpreted as a multiplicity of genes, and their size and number can be calculated from the inactivation doses

of the different radiations. The principle of this calculation is as follows. If there are N spherical targets of diameter $2r$ in a phage particle, ionization in any one of which inactivates the phage, then the inactivation dose of the phage will be $1/N$ times the inactivation dose for a single spherical target of diameter $2r$. The latter may be read off for any value of $2r$ from the calibration curves already referred to. By trial one finds values of N and $2r$ which yield inactivation doses which approximate to the experimentally measured inactivation doses. It is seen in table 6 that the experimental data are satisfactorily fitted by the assumption that phage C-36 contains nine targets (i.e. on our interpretation, nine genes) of diameter $10\text{ m}\mu$, and that phage Staph-K contains fourteen targets of diameter $12.5\text{ m}\mu$.

TABLE 6. COMPARISON OF EXPERIMENTAL INACTIVATION DOSES WITH THOSE CALCULATED ON THE ASSUMPTION THAT INACTIVATION IS LETHAL MUTATION

phage	postulated gene diam. $\text{m}\mu$	postulated gene number		inactivation doses in millions of roentgens		
				γ -rays	X-rays	α -rays (Po)
C-36	10	9	experimental	0.21	0.43	0.94
			calculated	0.22	0.36	1.01
Staph-K	12.5	14	experimental	0.079	0.109	0.45
			calculated	0.075	0.129	0.41

Thus phages C-36 and Staph-K appear to be very elementary organisms, as judged by the small number of genes we have estimated them to contain. Vaccinia virus is more complex, as judged by its larger number of genes (about one hundred, Lea 1946, revising the estimate of Lea & Salaman 1942), but not so complex as animal cells, which are generally believed to contain some thousands of genes.

We are indebted to the British Empire Cancer Campaign for defraying the cost of X-ray equipment, to the Medical Research Council and the National Radium Commission for the loan of radium, to Professor S. Russ and Mr G. W. Spicer for radon and old radon seeds. We would also like to thank Dr W. J. Elford who supplied the bacteriophages and the appropriate sensitive strains of bacteria. M. H. S. is indebted to the Director of the Strangeways Laboratory for hospitality and working facilities.

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The electrical constants of a crustacean nerve fibre

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Theoretical equations are derived for the response of a nerve fibre to the sudden application of a weak current. The equations describe the behaviour of the nerve fibre in terms of the membrane resistance and capacity, the axoplasm resistance and the resistance of the external fluid. Expressions are given which allow these four constants to be calculated from experimental observations.

Axons from *Carcinus maenas* were used in preliminary experiments. Quantitative determinations were made on a new single-fibre preparation—the 75μ diameter axon from the walking leg of the lobster (*Homarus vulgaris*). Currents with a strength of one-third to one-half threshold were used in the quantitative determinations.

The behaviour of lobster axons agreed with theoretical predictions in the following respects: (a) the steady extrapolar potential declined exponentially with distance; (b) the voltage gradient midway between two distant electrodes was uniform; (c) the rise and fall of the extrapolar potential at different distances conformed to the correct theoretical curves.

The extrapolar potential disappeared when the axon was treated with a solution of chloroform, indicating that the surface membrane was destroyed by this treatment, and that the potential recorded was in fact derived from the membrane.

The ratio of the internal to external resistance per unit length was found to be about 0.7.

The absolute magnitude of the action potential at the surface membrane was estimated at about 110 mV.

The specific resistance of the axoplasm had an average value of 60Ω cm., which was roughly three times that of the surrounding sea water.

The calculated resistance of one square centimetre of membrane was found to vary from 600 to 7000 Ω in thirteen experiments.

The membrane capacity was of the order of $1.3\mu\text{F cm.}^{-2}$.

No trace of inductive behaviour could be observed in the majority of the experiments. But three axons with low membrane resistances showed effects which could be attributed either to inductance or to a small local response. The absence of inductive behaviour in axons with high membrane resistance does not prove the absence of an inductive element. Currents with a strength several times greater than threshold often produced oscillating potentials at the cathode.

A local response was always observed when the strength of current approached threshold. The response had a striking inflected form if the current strength was near threshold and its duration less than the utilization time.

Indirect evidence indicates that the membrane resistance falls to a low value during activity.

Experiments with non-medullated nerve fibres have shown that a sub-threshold electric current produces two quite distinct effects (Hodgkin 1938; Pumphrey, Schmitt & Young 1940). Currents with a strength less than half-threshold produce a voltage which behaves as though it were due to the passive accumulation of charge at the nerve membrane. This voltage varies linearly with the applied current and is sometimes called an electrotonic potential. Currents with a strength greater than half-threshold evoke an additional wave of negativity which is non-linear and which behaves as though it were a subliminal response in the cathodic part of the nerve fibre. The present paper is concerned with an analysis of the first of these effects and contains only qualitative observations of the second. There are several reasons for believing in the importance of such an analysis. In the first place a physical understanding of the passive behaviour of nerve is essential to any theory of excitation. Thus a strength-duration curve cannot be explained until the time course of the voltage across the excitable membrane is known. Nor can the mechanism of excitation by the action potential be fully understood until there exists a thorough knowledge of the effect of applied currents on a single nerve fibre. A physical analysis is also interesting because it provides an insight into the structure of the surface membrane. Physical chemists are now able to prepare very thin films of lipoid material between two aqueous phases (Dean 1939), and it is clearly of the utmost importance to compare the electrical resistance and capacity of such films with those of the surface membrane in the living cell. The membrane resistance is also interesting from a more general point of view. Many biological processes depend upon the movement of ions through cell membranes, and the rate at which ions are transferred across a membrane should be related to its electrical resistance. Our results may, therefore, be of use to those who study the ionic movements that occur in the processes of growth, secretion and respiration. But perhaps the most important reason for making an analysis of the passive properties of a nerve fibre is that such an analysis must precede an understanding of the more complicated electrical changes which make up the nervous impulse itself.

Certain assumptions about the electrical structure of a nerve fibre must be made before any analysis can be started. The basic assumption of our work is that the structure of a non-medullated nerve fibre is similar to that of other cells which are known to have an interior of conducting protoplasm and a thin surface membrane with a high leakage resistance and a large capacity per unit area (Höber 1910; Fricke & Morse 1925; Cole 1937, etc.). If this general type of structure is granted, it follows that the passive behaviour of a nerve fibre must be governed by the equations of cable theory (Cremer 1899; Hermann 1905; Rushton 1934; Bogue & Rosenberg 1934; Cole & Curtis 1938, and others). The quantitative behaviour of the fibre should be determined by four electrical constants, viz.

- (1) The electrical resistance of the fluid outside the nerve fibre.
- (2) The electrical resistance of the axoplasm.
- (3) The electrical capacity of the surface membrane.
- (4) The electrical resistance of the surface membrane.

One other parameter, the membrane inductance, may have to be added (Cole 1941), but will not be considered in the initial stages of this paper. There already exists a considerable amount of information about the magnitude of three of these quantities. The external resistance can be calculated from the volume and conductivity of the fluid bathing the nerve fibre; the cell interior appears to have a resistivity two or three times as great as that of the external fluid and the surface membrane to have a capacity of about $1 \mu\text{F}/\text{sq.cm.}$ Very little is known about the membrane resistance and measurements have so far been confined to a few plant cells (Blinks 1937) and one animal cell, the giant nerve fibre of the squid (Cole & Hodgkin 1939; Cole 1940).^{*} The determination of the membrane resistance was therefore the first aim of our experiments and measurement of the remaining constants was originally regarded as of secondary importance. But it so happens that one constant cannot be determined without making measurements of at least two others. An attempt was therefore made to determine all four quantities simultaneously on a single fibre. Four sets of measurements had to be made since there were four unknowns to be evaluated, and after several trials we chose the following methods:

- (1) The extent of spread of potential in the extrapolar region.
- (2) The rate of rise of potential in the extrapolar region.
- (3) The ratio of the applied current to the voltage recorded between cathode or anode and a distant extrapolar point.
- (4) The voltage gradient in the region midway between two distant electrodes.

Axons with a diameter of 30μ from *Carcinus maenas* (Hodgkin 1938) were used initially. This work served to develop the experimental technique, but the extent of spread of potential was thought too small for accurate measurement. A search was therefore made for a fibre with a larger diameter and a suitable preparation was eventually found in the walking legs of the lobster (*Homarus vulgaris*). The meropodite of the walking legs contains a few fibres which have a diameter of 75μ and are robust enough to permit isolation without damage. This preparation was used in the majority of experiments. Electrical measurements were made by applying rectangular pulses of current and recording the potential response photographically. About fifteen sets of film were obtained in May and June of 1939, and a preliminary analysis was started during the following months. The work was then abandoned and the records and notes stored for six years. A final analysis was made in 1945 and forms the basis of this paper. A certain amount of biophysical work has proceeded during the interval, but no one seems to have repeated these particular experiments.

NOMENCLATURE

The passive spread of potential which occurs in nerve fibres is sometimes described by the term polarization potential and sometimes electrotonus or electrotonic potential. Both words are unfortunate. Electrotonus implies that the

^{*} An estimate of the plasma membrane resistance in the frog's egg has been made by Cole & Guttman (1942).

axon is in a state of enhanced physiological activity: polarization potential that the change of voltage is due to an alteration of ionic concentration in the vicinity of the membrane. Weak currents do not necessarily evoke an active or tonic response; nor is it at all certain that there is a significant polarization in the sense of Nernst (1908) or Warburg (1899). For it seems likely that the change of voltage with current is due to a frictional resistance opposing the motion of ions and not to changes in ionic concentration. We have therefore avoided the use of both terms so far as is possible and have used instead words such as membrane potential or extrapolar potential according to the context.

Wherever possible we have used the same symbols as Cole and his colleagues. λ has been employed as a space constant and should not be confused with the λ of Hill's theory of excitation (Hill 1936). The dual use of symbols is unfortunate but cannot be avoided, since both American and British writers have used λ as a space constant (e.g. Cole & Curtis 1938; Rushton 1934).

THEORETICAL SECTION

Assumptions

(1) The axon has a uniform cable-like structure with a conducting core, an external conducting path and a surface membrane with resistance and capacity.

(2) The axon is sufficiently thin and the membrane resistance sufficiently high for the flow of current in core and interstitial fluid to be strictly parallel. An alternative statement of this assumption is that at any given distance along the nerve the potential is constant throughout the core or throughout the external fluid.

(3) The axoplasm and external fluid behave as pure ohmic resistances.

(4) The membrane resistance is constant when the current density through the membrane is small.

(5) The membrane capacity behaves like a pure dielectric with no loss.

These are general assumptions which allow the differential equations for current or potential to be written. Each assumption is really an approximation, but we shall show later that no very serious errors are likely to result from their use. Certain experimental conditions must also be defined in order to allow the differential equations to be solved. These may be stated in the following way:

(1) The extrapolar and interpolar lengths are sufficiently long to be taken as infinite.

(2) The electrodes are sufficiently fine to be considered of zero breadth.

(3) A current of rectangular wave form is passed through the nerve.

Symbols and definitions

Variables

x is distance along axon in cm.

t is time in seconds.

i_1 is the current in amperes flowing through the external fluid (figure 1).

i_2 is the current in amperes flowing through the axis cylinder.

I is the total current in amperes flowing through the fibre and external fluid
($I = i_1 + i_2$).

i_m is the current penetrating the surface membrane at any point in ampere cm.⁻¹.

V_1 is the potential in volts of the external fluid with respect to a distant point
($V_1 = -\int_{-\infty}^x r_1 i_1 dx$).

V_2 is the potential in volts of the axis cylinder with respect to a distant point
($V_2 = -\int_{-\infty}^x r_2 i_2 dx$).

V_m is the change in potential difference across the surface membrane which results from the flow of current ($V_m = V_1 - V_2$).

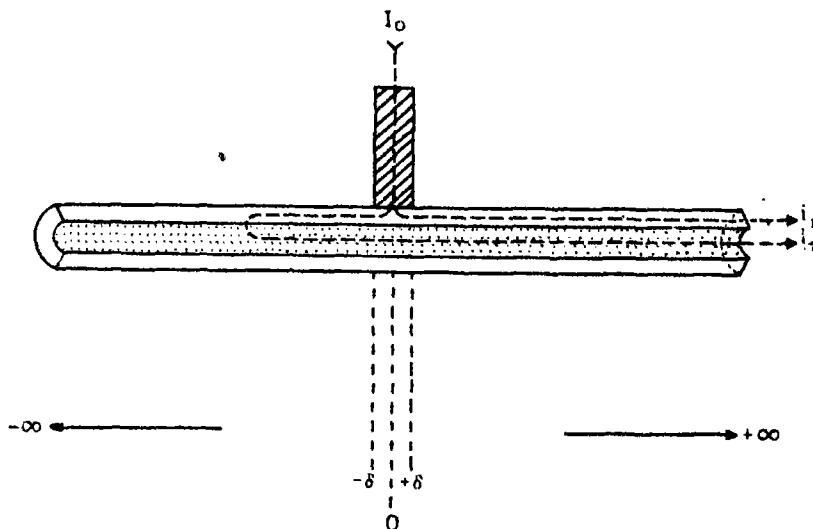


FIGURE 1. Geometry of system considered in theoretical section.

Basic constants

a is the radius of the axis cylinder in cm.

R_2 is the specific resistivity of the axoplasm in Ω cm.

R_4 is the resistance \times unit area of the surface membrane in Ω cm.².

C_M is the capacity per unit area of the surface membrane in F cm.⁻².

Practical constants

r_1 is the resistance per unit length of the external fluid in Ω cm.⁻¹.

r_2 is the resistance per unit length of the axis cylinder in Ω cm.⁻¹ ($r_2 = R_2/\pi a^2$).

r_4 is the resistance \times unit length of the surface membrane in the axon in Ω cm.
($r_4 = R_4/2\pi a$).

c is the capacity per unit length of the surface membrane in the axon in F cm.⁻¹
($c = C_M \times 2\pi a$).

$\lambda = \sqrt{(r_4/r_1 + r_2)}$, and is the characteristic length in cm.

$m = r_1 r_2 / r_1 + r_2$, and is the parallel resistance of the axis cylinder and external fluid in Ω cm.⁻¹.

$y = m\lambda r_1 / 2r_2 = r_1^2 \lambda / 2(r_1 + r_2)$.

$\tau_m = r_4 c = R_4 C_M$, and is the characteristic time of the surface membrane in seconds.

Miscellaneous

δ is equal to half the electrode width (figure 1). This quantity will eventually be made vanishingly small, but is introduced in order to deal with discontinuities. X , T , U , ξ and I_0 are best defined as they are introduced.

Theory

A number of useful equations follow at once from the definitions:

$$\frac{\partial V_1}{\partial x} = -r_1 i_1, \quad (1.0)$$

$$\frac{\partial V_2}{\partial x} = -r_2 i_2, \quad (1.1)$$

$$\frac{\partial V_m}{\partial x} = (r_1 + r_2) i_2 - I r_1, \quad (1.2)$$

$$V_m = \left(\frac{r_1 + r_2}{r_1} \right) V_1 + r_2 \int_{-\infty}^x I dx. \quad (1.3)$$

In the extrapolar region $I = 0$ so that (1.3) can be simplified to

$$V_m = \left(\frac{r_1 + r_2}{r_1} \right) V_1. \quad (1.4)$$

The total current through the membrane can be obtained in two ways:

$$i_m = \frac{\partial i_2}{\partial x}, \quad (1.5)$$

$$i_m = \frac{V_m}{r_4} + c \frac{\partial V_m}{\partial t}. \quad (1.6)$$

Hence

$$\frac{V_m}{r_4} + c \frac{\partial V_m}{\partial t} = \frac{\partial i_2}{\partial x}, \quad (1.7)$$

and on substituting from (1.2)

$$\frac{V_m}{r_4} + c \frac{\partial V_m}{\partial t} = \frac{1}{r_1 + r_2} \frac{\partial^2 V_m}{\partial x^2} + \frac{r_1}{r_1 + r_2} \frac{\partial I}{\partial x}, \quad (2.0)$$

or

$$-\lambda^2 \frac{\partial^2 V_m}{\partial x^2} + \tau_m \frac{\partial V_m}{\partial t} + V_m = r_1 \lambda^2 \frac{\partial I}{\partial x}. \quad (2.1)$$

Now $\partial I/\partial x$ vanishes except at the electrode, since $I = 0$ for $-\infty < x < -\delta$ and $I = I_0$ for $\delta < x < \infty$. Hence the following equation (2.2) applies to the regions $-\infty < x < -\delta$, $\delta < x < \infty$:

$$-\lambda^2 \frac{\partial^2 V_m}{\partial x^2} + \tau_m \frac{\partial V_m}{\partial t} + V_m = 0. \quad (2.2)$$

This equation must now be solved for the particular case where I is a constant current I_0 starting abruptly at $t = 0$.

The boundary conditions are

$$V_m = 0 \text{ everywhere } -\infty < t < 0, \quad V_m = 0 \text{ always when } x = \pm \infty.$$

There are also two continuity conditions. First, V_m is always a continuous function of x , since a discontinuity in V_m would mean that an infinite current must flow through the nerve. Secondly, i_2 is also a continuous function of x since the current density through the membrane cannot be infinite when $t \neq 0$. Introduce new variables $X = x/\lambda$, $T = t/\tau_m$, $U = V_m e^T$. Equation (2.2) can now be written

$$-\frac{\partial^2 V_m}{\partial X^2} + \frac{\partial V_m}{\partial T} + V_m = 0, \quad (2.3)$$

$$\text{or} \quad -\frac{\partial^2 U}{\partial X^2} + \frac{\partial U}{\partial T} = 0. \quad (2.4)$$

The operator q^2 may be substituted directly for $\partial/\partial T$ since $U = 0$ when $T = 0$. Hence

$$\frac{\partial^2 U}{\partial X^2} = q^2 U. \quad (3.0)$$

The solutions of (3.0) are

$$U = A e^{qX} + B e^{-qX}, \quad \text{when } -\infty < X < -\delta/\lambda,$$

$$U = A_1 e^{qX} + B_1 e^{-qX}, \quad \text{when } \delta/\lambda < X < \infty.$$

But the second boundary condition indicates that $U \neq \infty$ when $X = \pm \infty$, so $B = 0 = A_1$.

From the first continuity condition it follows that $A = B_1$, since

$$U_{X=\delta/\lambda} = U_{X=-\delta/\lambda},$$

when δ/λ is made vanishingly small. Hence

$$U = A e^{qX} \quad \text{for } -\infty < X < -\delta/\lambda, \quad (3.1)$$

$$U = A e^{-qX} \quad \text{for } \delta/\lambda < X < \infty. \quad (3.2)$$

The value of A can be found by applying the continuity of i_2 to equation (1.2). For

$$\left(\frac{\partial V_m}{\partial x}\right)_{x=\delta} - \left(\frac{\partial V_m}{\partial x}\right)_{x=-\delta} = (r_1 + r_2) \{(i_2)_{x=\delta} - (i_2)_{x=-\delta}\} - r_1(I_{x=\delta} - I_{x=-\delta}),$$

$$\text{whence} \quad \left(\frac{\partial U}{\partial X}\right)_{X=\delta/\lambda} - \left(\frac{\partial U}{\partial X}\right)_{X=-\delta/\lambda} = -r_1 I_0 \lambda e^T,$$

which in the operational form becomes $-r_1 \lambda I_0 \frac{q^2}{q^2 - 1}$. But from (3.1) and (3.2)

$$\left(\frac{\partial U}{\partial X}\right)_{X=-\delta/\lambda} - \left(\frac{\partial U}{\partial X}\right)_{X=-\delta/\lambda} = -2qA.$$

So
$$U = \frac{r_1 \lambda I_0}{4} \left\{ \frac{1}{q-1} + \frac{1}{q+1} \right\} e^{qX} \quad \text{for } -\infty < X < -\delta/\lambda, \quad (3.3)$$

and
$$U = \frac{r_1 \lambda I_0}{4} \left\{ \frac{1}{q-1} + \frac{1}{q+1} \right\} e^{-qX} \quad \text{for } \delta/\lambda < X < \infty. \quad (3.4)$$

The interpretations of the operational expressions in (3.3) and (3.4) are known (Jeffreys 1931). When they are substituted, the following equations for V_m are obtained:

$$V_m = \frac{r_1 \lambda I_0}{4} \{ e^X [1 + \operatorname{erf}(X/2\sqrt{T} + \sqrt{T})] - e^{-X} [1 + \operatorname{erf}(X/2\sqrt{T} - \sqrt{T})] \},$$

when $-\infty < X < 0$, (4.0)

and
$$V_m = \frac{r_1 \lambda I_0}{4} \{ e^{-X} [1 - \operatorname{erf}(X/2\sqrt{T} - \sqrt{T})] - e^X [1 - \operatorname{erf}(X/2\sqrt{T} + \sqrt{T})] \},$$

when $0 < X < \infty$, (4.1)

where
$$\operatorname{erf} Z = \frac{2}{\sqrt{\pi}} \int_0^Z e^{-\omega^2} d\omega.$$

These expressions satisfy equation (2.3) and the boundary conditions. Campbell & Foster (1931, p. 162) give an expression which is equivalent to (4.1) for the response of a non-inductive cable to the sudden application of current.

The solutions for the case when the applied current is maintained for a long time and then broken suddenly at $t = 0$ can be written down at once from the superposition theorem. They are

$$V_m = \frac{r_1 \lambda I_0}{4} \{ e^X [1 - \operatorname{erf}(X/2\sqrt{T} + \sqrt{T})] + e^{-X} [1 + \operatorname{erf}(X/2\sqrt{T} - \sqrt{T})] \},$$

when $-\infty < X < 0$, (4.2)

and
$$V_m = \frac{r_1 \lambda I_0}{4} \{ e^{-X} [1 + \operatorname{erf}(X/2\sqrt{T} - \sqrt{T})] + e^X [1 - \operatorname{erf}(X/2\sqrt{T} + \sqrt{T})] \},$$

when $0 < X < \infty$. (4.3)

Equations (4.0), (4.1) and (4.2), (4.3) are symmetrical pairs differing only in the sign of X . Thus it is necessary to compute only one set of curves in order to describe the distribution of potential for the make or break of a constant current. And the curves for the break of current can be obtained from those for the make by a direct application of the superposition theorem. Equation (4.1) is the most convenient to compute, since it deals with positive values of X . An evaluation of the essential part of (4.1) is given in table 1 and the results are plotted graphically in figure 2.

TABLE 1. TABLE OF THE FUNCTION $\{e^{-X}[1 - \operatorname{erf}(X/2\sqrt{T} - \sqrt{T})] - e^X[1 - \operatorname{erf}(X/2\sqrt{T} + \sqrt{T})]\}$
FOR DIFFERENT VALUES OF X AND T

X	$T=0.01$	0.04	0.16	0.36	0.64	1.0	1.44	1.96	2.56	3.24	4.00	6.25	∞
0	0.2249	0.4454	0.8567	1.208	1.484	1.685	1.821	1.904	1.953	1.987	1.991	1.999	2.000
0.1	0.0795	0.2743	0.673	1.020	1.294	1.496	1.631	1.714	1.763	1.788	1.801	1.800	1.810
0.2	0.0200	0.1561	0.5201	0.855	1.126	1.325	1.459	1.542	1.590	1.616	1.628	1.636	1.637
0.3	0.0035	0.0816	0.3026	0.712	0.976	1.172	1.305	1.387	1.435	1.460	1.473	1.481	1.482
0.4	0.0004	0.0390	0.2921	0.588	0.842	1.034	1.165	1.247	1.294	1.320	1.332	1.340	1.341
0.5	—	0.0170	0.2125	0.483	0.725	0.911	1.040	1.120	1.167	1.192	1.204	1.212	1.213
0.6	—	0.0067	0.1513	0.392	0.621	0.801	0.926	1.006	1.052	1.077	1.089	1.097	1.098
0.7	—	0.0024	0.1055	0.316	0.530	0.702	0.824	0.903	0.948	0.972	0.984	0.992	0.993
0.8	—	0.00076	0.0718	0.252	0.450	0.614	0.732	0.809	0.853	0.878	0.890	0.898	0.899
0.9	—	—	0.0474	0.2000	0.381	0.537	0.651	0.725	0.769	0.793	0.804	0.812	0.813
1.0	—	—	0.0311	0.1554	0.319	0.467	0.577	0.649	0.691	0.715	0.727	0.735	0.736
1.2	—	—	0.0122	0.0937	0.224	0.351	0.451	0.517	0.560	0.583	0.594	0.601	0.602
1.5	—	—	0.0026	0.0401	0.125	0.225	0.309	0.369	0.407	0.428	0.438	0.445	0.446
2.0	—	—	0.0001	0.0086	0.0435	0.1008	0.159	0.205	0.236	0.254	0.263	0.270	0.271
2.5	—	—	—	0.0012	0.0129	0.0415	0.0773	0.1113	0.1347	0.1494	0.1574	0.1636	0.1642
3.0	—	—	—	—	0.0033	0.0174	0.0364	0.0581	0.0754	0.0870	0.0937	0.0988	0.0996

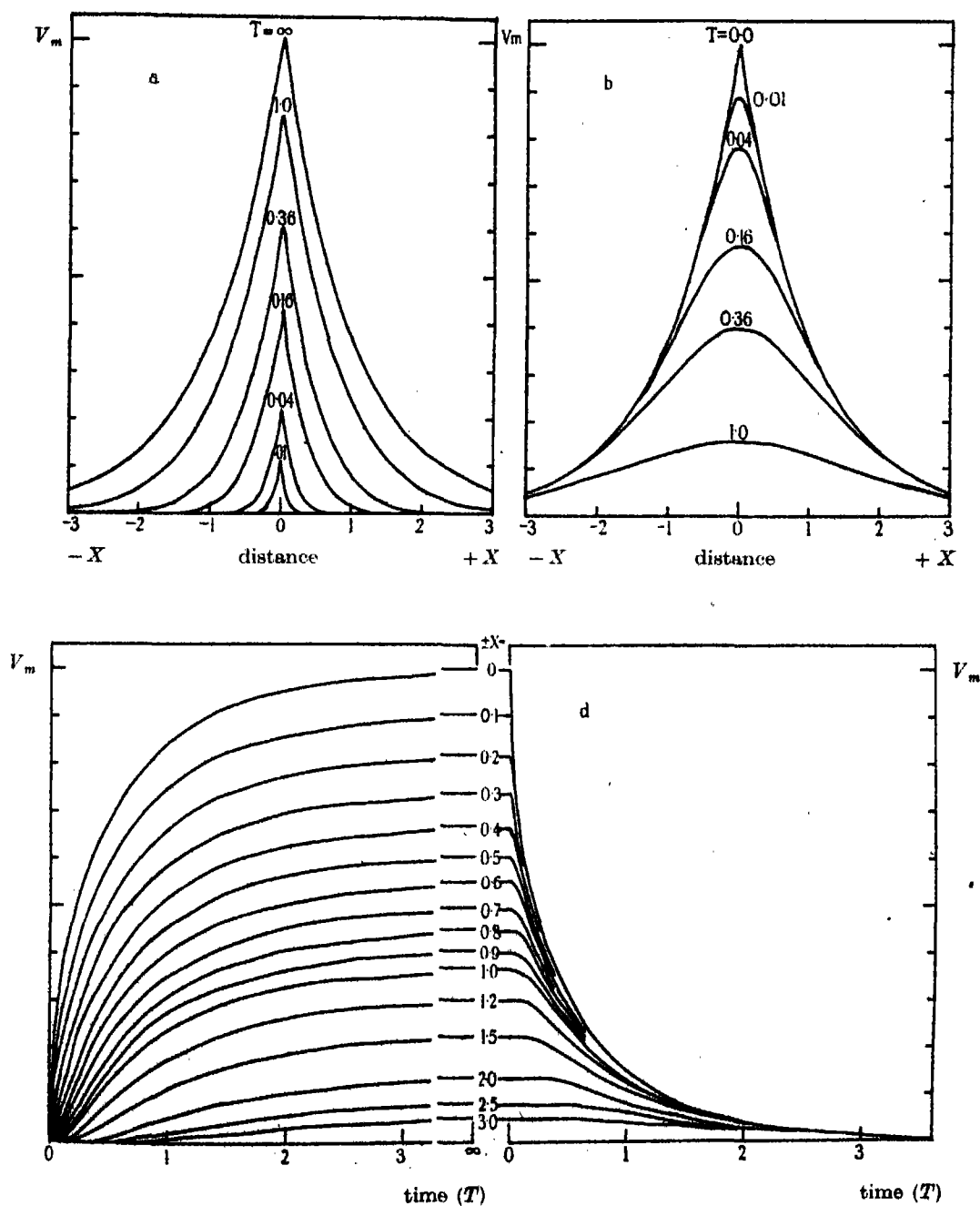


FIGURE 2. Theoretical behaviour of potential difference across nerve membrane (V_m). *a, b*, spatial distribution of potential at different times; *c, d*, time course of potential at different distances from electrode; *a, c*, current made at $T=0$; *b, d*, current maintained for a long time and then broken at $T=0$.

A great simplification of the mathematical theory can be achieved by considering the total charge in the region of the electrode instead of the membrane potential. Define total charge by a new variable

$$\xi = c \int_{-\infty}^{\beta} V_m dx, \quad (5.0)$$

where β is sufficiently large to allow the integration to include all the charge in the electrode region. β can be considered as infinite provided that the integration does not include the region of the second electrode. Integration of equation (2.1) from $-\infty$ to β gives

$$\tau_m \frac{\partial \xi}{\partial t} + \xi = r_1 c \lambda^2 I_0, \quad (5.1)$$

since
$$\int_{-\infty}^{\beta} \frac{\partial V_m}{\partial t} dx = \frac{\partial}{\partial t} \left[\int_{-\infty}^{\beta} V_m dx \right]$$

and $\frac{\partial V_m}{\partial x} = 0$ when $x = -\infty$ and $x = +\beta$. The solutions of (5.1) are

$$\xi = r_1 c \lambda^2 I_0 (1 - e^{-t/\tau_m}) \quad (5.2)$$

for a constant current made at $t = 0$ and

$$\xi = r_1 c \lambda^2 I_0 e^{-t/\tau_m} \quad (5.3)$$

for a constant current broken at $t = 0$. Unfortunately, these simple equations are of little practical use, since ξ can only be obtained indirectly from the experimental results.

The extrapolar potential

Equations (4.0) and (4.2) can be applied directly to the experimental results since $V_m = \frac{r_1 + r_2}{r_1} V_1$, $X = x/\lambda$ and $T = t/\tau_m$.

The most convenient expressions for the make of current are

$$V_1 = (V_1)_{t=\infty} \frac{1}{2} \{ e^X [1 + \operatorname{erf}(X/2\sqrt{T} + \sqrt{T})] - e^{-X} [1 + \operatorname{erf}(X/2\sqrt{T} - \sqrt{T})] \}, \quad (6.0)$$

where
$$(V_1)_{t=\infty} = \frac{r_1^2 \lambda I_0}{2(r_1 + r_2)} = y I_0, \quad (6.1)$$

y being thus defined in the table of practical constants,

$$(V_1)_{t=\infty} = (V_1)_{t=\infty} e^X, \quad (6.2)$$

and
$$(V_1)_{x=0} = (V_1)_{t=\infty} \operatorname{erf}(\sqrt{T}). \quad (6.3)$$

For the break of current the relevant expressions are

$$V_1 = (V_1)_{t=\infty} \frac{1}{2} \{ e^X [1 - \operatorname{erf}(X/2\sqrt{T} + \sqrt{T})] + e^{-X} [1 + \operatorname{erf}(X/2\sqrt{T} - \sqrt{T})] \}, \quad (6.4)$$

and
$$(V_1)_{x=0} = (V_1)_{t=\infty} [1 - \operatorname{erf}(\sqrt{T})]. \quad (6.5)$$

The mid-interpolar gradient

The expressions given in the preceding paragraph allow λ , τ_m and y to be determined from experimental observations of the extrapolar potential. The constant m can be obtained from a measurement of the voltage gradient in the interpolar region at a large distance from either electrode. For equation (4.1) shows that

$\left(\frac{\partial V_m}{\partial x}\right)_{x=\beta} = 0$ when $\beta \gg \lambda$. Hence differentiation of (1.3) gives

$$-\left(\frac{\partial V_1}{\partial x}\right)_{x=\beta} / I_0 = \frac{\tau_1 r_2}{\tau_1 + r_2} = m. \quad (7.0)$$

Determination of basic constants

Convenient expressions for determining the basic constants are

$$R_2 = \pi a^2 m (1 + m\lambda/2y), \quad (8.0)$$

$$R_4 = 2\pi a\lambda^2 m (2 + m\lambda/2y + 2y/m\lambda), \quad (8.1)$$

$$C_M = \tau_m / R_4. \quad (8.2)$$

An expression for the resistivity of the external fluid is not given because there was no easy way of determining the volume of fluid surrounding the nerve fibre. Nor would this quantity have been of great interest. But it is desirable to have an index of the amount of short-circuiting introduced by the external fluid and the ratio r_2/r_1 has been used for this purpose. It can be computed by the relation

$$r_2/r_1 = m\lambda/2y. \quad (8.3)$$

VALIDITY OF ASSUMPTIONS

We are now in a better position to assess the errors which are introduced by the approximations made in the theory. The assumption of parallel current flow is not likely to involve any serious error provided that the current spreads over a length which is several times greater than the diameter of the axon. This condition was satisfied experimentally, since the average value for the space constant λ was twenty times greater than the axon diameter. The assumption of zero breadth for the electrode can be justified in the same way, since λ/δ was also of the order of twenty. Both approximations are doubtful at short time intervals. Thus figure 2 shows that the effective space constant is only $\lambda/5$ when t/τ_m is 0.04. But it can be said that the cable equations apply with reasonable accuracy provided $t/\tau_m > 0.04$.

In practice anode and cathode were separated by about 8 mm. of nerve; theory assumes them to be an infinite distance apart. But interference between the two electrode regions must have been negligible, since 8 mm. was equivalent to 5λ in an average experiment and e^{-5} is 0.007. A similar argument applies to the recording electrodes which were also 8 mm. apart.

The assumption that the internal and external resistances obey Ohm's law is fully justified by earlier work (see, for instance, Cole & Hodgkin 1939) and finds

further confirmation in the measurements of mid-interpolar gradient which will be described presently. The constancy of the membrane resistance might be questioned in view of Cole & Curtis's (1941) demonstration of the rectifying properties of the surface membrane. But any rectifier behaves as a linear element if it is examined with a sufficiently weak current. And we shall show later that the measuring currents used were probably small enough to keep the membrane in a linear part of its characteristic.

Some error must have been introduced by assuming that the membrane capacity behaved like a pure dielectric. The magnitude of the error cannot be estimated in any simple way, but it is not likely to have been very large. For a.c. measurements give a value of 76° for the phase angle of the dielectric of the membrane in the squid axon (Curtis & Cole 1938). This suggests that the membrane capacity would be reduced by 30 % when the frequency was increased tenfold. Most of the records dealt with here could be reproduced fairly accurately by a Fourier synthesis containing a tenfold range of frequencies and so would not have been greatly affected by imperfections in the membrane capacity.

METHOD

Material

Single-nerve fibres with a diameter of $60\text{--}80\mu$ were obtained from the walking legs of the common lobster (*Homarus vulgaris*). Live lobsters were bought from a fishmonger and kept in an aquarium filled with circulating sea water. The animals were in poor condition when first obtained, but they recovered after a few hours in the aquarium and were able to live there for several weeks. Axons were obtained from the first two pairs of walking legs which are chelate and appear to be better supplied with large fibres than the last two which have no terminal claw. The nerve was dissected from the meropodite and teased apart in a Petri dish of sea water. *Homarus* nerve contains much connective tissue, and separation of a single fibre proved to be a more laborious process than in a *Carcinus* preparation. More time had to be spent in cutting away connective tissue, and no attempt could be made to pull fibres apart until they had been freed from the strands of connective tissue which bound them together. All loose material was removed from the isolated axon whose length varied from 25 to 40 mm. Fibres with branches were never employed.

The method of isolating *Carcinus* axons was similar to that employed in earlier work (Hodgkin 1938) and need not be described again.

Apparatus

A general plan of the equipment used is shown in figure 3. The axon was kept in paraffin oil and was gripped at each end by the tips of insulated forceps (*AA'*). It was held in a horizontal position and could be observed from above by means of a binocular microscope. The axon rested on the wick electrodes *B*, *D*, and made contact with the tip of electrode *C*. Electrodes *B* and *D* made contact over a length of about 250μ , and electrode *C* over approximately 100μ . These three electrodes consisted of small glass tubes containing sea water and silver wires which had been coated electrolytically with chloride. One end of the glass tube was sealed with wax; the other was drawn out into a coarse capillary and plugged with agar sea water. Connexion to the nerve was made through fine agar wicks which projected for about 5 mm. beyond the tip of the glass capillary. The wicks were built by allowing agar sea water to solidify around a fine silk thread. Silver chloride electrodes were sufficiently non-polarizable, since a $5\text{M}\Omega$ resistance in series with the electrodes ensured that the current was entirely unaffected by residual electrode polarization. Electrode *E* did not need to be non-

polarizable, since it was used only for recording transient pulses with an amplifier of high input impedance. This electrode consisted of a fine glass tube into which a platinum wire was sealed; one end of the tube was drawn out into a fine glass capillary, ground square and the whole filled with sea water. The diameter of the tip was about 50μ and the region of contact with the nerve fibre of the same order of magnitude.

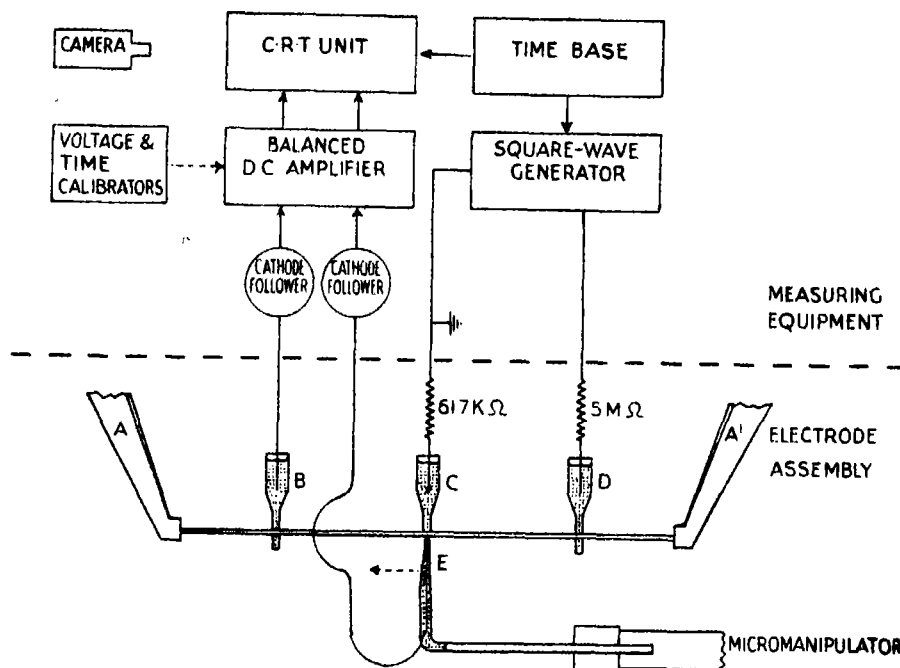


FIGURE 3. General plan of equipment. For letter references, see text.

Electrode *E* was held in a micromanipulator carriage and could be moved along the nerve fibre by turning one of the vernier controls on the manipulator. The electrode slid smoothly along the fibre provided that all loose connective tissue had been removed and that the direction of movement was parallel to the axis of the fibre. The position of the electrode was determined by a scale on the manipulator which was calibrated to read in fractions of a millimetre. This method of measurement was checked periodically by observing the motion of the electrode under the binocular microscope. The movement of the electrode was found to be the same as that given by the scale on the screw adjustment. Back-lash could be taken as zero, since it was less than 10 micra.

All electrical measurements were made by applying a rectangular pulse of current to the axon and recording the resulting potential changes with an amplifier and oscillograph. The rectangular pulse was generated by means of an arrangement of thyratrons (R.C.A. 885) and a multivibrator of the type described by Schmitt (1938). The wave form of the rectangular pulses was tested by connecting the pulse output to the plates of a cathode-ray tube. This showed that the deflexion was 90 % complete in less than $10\mu\text{sec}$. The pulse could be synchronized with the sweep circuit and its duration varied between 10 and $10^6\mu\text{sec}$. A low-resistance attenuator was used for varying the magnitude of the pulse applied to the nerve. One terminal of the pulse generator was connected to earth; the other became positive for the duration of the pulse. The positive-going terminal was connected to electrode *D* through $5\text{M}\Omega$ and the other terminal (earth) to electrode *C* through a monitoring resistance of $61,700\Omega$. The 5-megohm resistance ensured that a constant current was passed through the nerve, while the monitoring resistance was used to measure the current through the nerve fibre. The pulses of current were repeated at a rate of about one a second.

Electrical changes were recorded with a balanced d.c. amplifier designed by Dr Rawdon Smith of the Psychological Laboratory, Cambridge. This consisted of three pairs of pentodes with separate anode loads and common cathode resistances. The line voltages were arranged so that the anode of one stage could be connected to the grid of the next. In this way the undesirable resistance chains usually associated with d.c. amplifiers were avoided. Occasional checks showed that the differential action of the amplifier was better than one part in five hundred (i.e. when both inputs were raised 1 V above earth the oscillograph deflexion was equivalent to less than 2 mV difference between inputs). Initial checks with a signal generator indicated that the response of the amplifier was substantially flat between 0 and 50 kcyc./sec. In order to increase the input impedance the recording leads were connected to the grids of two cathode followers which were placed at a distance of 15 cm. from the preparation. Calibrations of the input stage and the whole amplifier were made by applying the rectangular pulse to the grids of the input stage through a resistance of the same magnitude as that involved in recording from the nerve. This test showed that the deflexion produced was 90 % complete in about $30\mu\text{sec.}$, and the system was therefore sufficiently rapid for the investigation of phenomena lasting several milliseconds. The d.c. input impedance was greater than $10^{10}\Omega$ and the grid current less than 10^{-10} amp.

The time base was calibrated by applying the output from a 500 cyc./sec. oscillator to the amplifier. Voltage calibrations were made by photographing the series of oscillograph lines produced by varying the position of a decade resistance attenuator. In this way a calibration grid was obtained and could be compared with the experimental results. In general the experimental records fell in a region which was linear to within 2 % and so could be analysed without correction. Corrections had occasionally to be made but did not materially affect the results, since the amount of instrumental distortion rarely exceeded 5 %. All photographic records were taken on film and were traced on to graph paper after they had been enlarged about ten times.

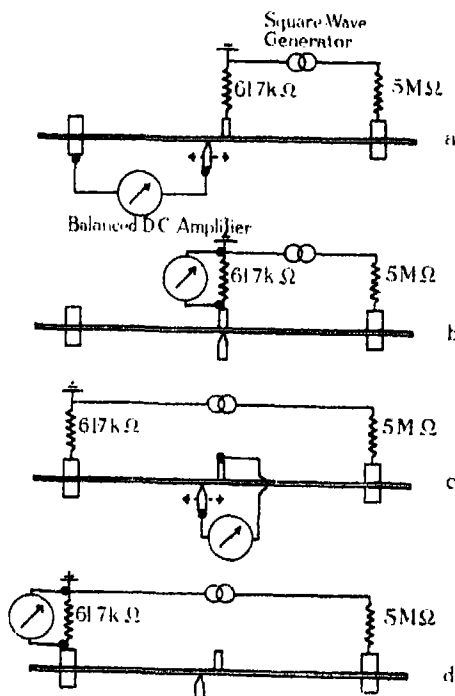


FIGURE 4. Arrangement of leads and electrodes employed in a quantitative experiment. *a*, system used for determining λ and τ_m ; *b*, system used in conjunction with *a* for determining y ; *c*, *d*, system used for determining m .

A typical experiment

The sequence of events in a quantitative experiment must now be described. The isolated axon was mounted on the electrode system and raised into a layer of aerated mineral oil which floated on the surface of the sea water. The recording electrode was brought into contact with the axon and a preliminary test made to ensure that the action potential was propagated normally throughout the whole fibre. The strength of current was reduced until it was below half-threshold and the potential response observed visually on the C.R.T. The duration of the rectangular wave was adjusted until it was sufficient to allow the membrane voltage to reach its equilibrium value. A test was made to ensure that the recording electrode slid smoothly along the axon. A series of photographic records of the extrapolar potential was then obtained with the arrangement of electrodes shown in figure 4*a*; in general these were similar to those in figure 6. One set of records was made with the movable electrode receding from the cathode and another with it approaching. There was sometimes a difference of 5 or 10 % between the two sets of records, but as a rule they agreed closely with one another. The current through the axon was determined with the arrangement of electrodes shown in figure 4*b* and a typical record is given in figure 6. This observation also provided a routine check of the squareness of the current wave form through the nerve fibre. The next operation was to determine the voltage gradient in the mid-interpolar stretch using the arrangement of figure 4*c*. The recording electrode was moved along the axon and a series of records similar to those in figure 9*a* obtained. The current through the axon was again determined; the arrangement of leads being that of figure 4*d* and a typical record that of figure 9*b*. At the end of each experiment the fibre diameter was measured in the following way. The axon was lowered into sea water and transferred to a hollow-ground slide; it was then examined with a microscope using a $\frac{1}{4}$ in. objective and an eyepiece micrometer. Some variation in diameter was always encountered, but this rarely exceeded 5 %.

RESULTS

*Preliminary experiments**Local response and passive spread of potential*

In attempting to measure the membrane resistance it is important to ensure that measurements are made in the linear part of the nerve characteristic, and that the results are not complicated by the non-linear phenomena of local response and rectification. From this point of view currents which are much weaker than threshold should be employed. On the other hand, as the current is reduced the amplification must be increased and errors from other sources increase. This fact will be appreciated by anyone who has worked with a single-fibre preparation and a high-gain d.c. amplifier. It is sufficient to mention the difficulties which arise from stray interference, shock artifact and the irregular drifts in voltage which occur in the amplifier and in the nerve and electrode system. Preliminary tests indicated that a reasonable compromise would be to use currents with a strength of 0.4–0.5 threshold. An absolute value for the resulting current density through the membrane cannot be given, since it varied with the excitability and membrane resistance of individual axons. But a rough estimate is that the current density under the electrode was of the order of $5 \mu\text{A cm.}^{-2}$. The total current through the axon was roughly $0.1 \mu\text{A}$. The absence of any significant response in the region below half-threshold is illustrated by an experiment with a *Carcinus* fibre (figure 5). Here the behaviour of the axon is shown for different strengths of applied current. Anodic or weak cathodic currents appear to affect only the passive charging process;

for all the curves have the same shape and their amplitude is roughly proportional to the applied current. And the shape of the curves is of a type which is to be expected from a process involving passive charge and discharge of the membrane capacity. The picture changed completely when the applied current approached threshold. At 0.9 threshold the cathodic potential showed a fast creep, and at 1.0 the curves turned upwards as if to give rise to a propagated impulse. But a true

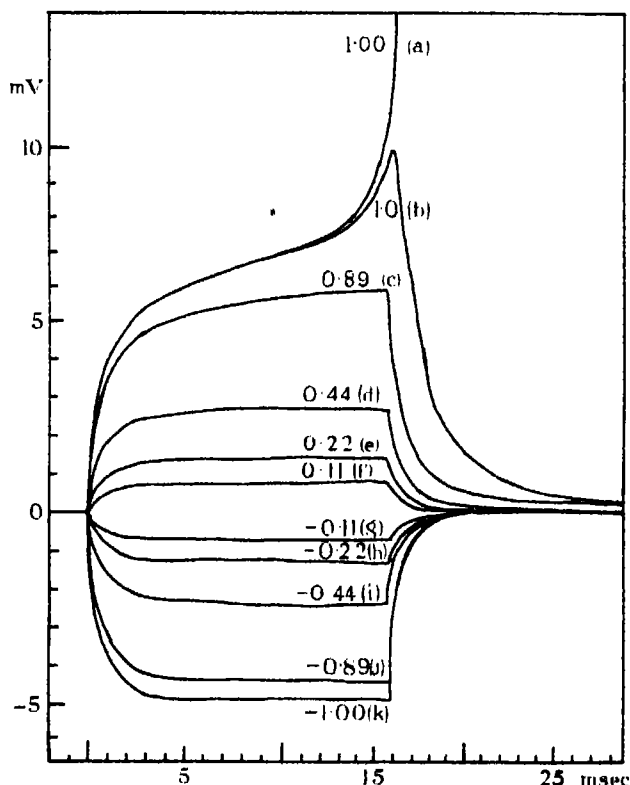


FIGURE 5. Response of *Carcinus* axon to rectangular waves of current of different intensity; recorded at a polarizing electrode of width about 200μ . The numbers on each record give the strength of current relative to threshold. Depolarization of the nerve is shown as positive.

action potential did not result in every case. Owing to the spontaneous play in excitability, a stimulus does not invariably evoke an impulse until its strength is slightly greater than threshold. In fact, a threshold shock is normally defined as one which produces impulses on 50 % of occasions. Record *b* shows what happens when a threshold shock failed to evoke an impulse. The potential turned upwards as if to give rise to a spike, but it failed to reach a critical level and died out as a localized wave. Inflected local responses of this kind only occurred when the current was nearly threshold but their onset was completely gradual. Thus all transitions between records *b* and *c* could be obtained by careful adjustment of the strength of current.

The striking form of local response illustrated by this experiment was observed on a large number of occasions and will be described in greater detail later. For the moment our chief concern is that it did not occur when the current was less than half-threshold.

Measurement of the curves in figure 5 indicated that there were small deviations from linearity in the region below half-threshold. At present there is no evidence to show whether these deviations were reproducible, and they may well have been instrumental in origin.

The effect of long pulses of current

The observations of Cole & Hodgkin (1939) on membrane resistance were made with currents lasting several seconds, while the duration of the currents used in the present work was of the order of 20 msec. It is legitimate to ask whether the two methods of measurement give comparable results. One or two experiments with long pulses were made in order to answer this question. The point at issue is whether the steady potential which is established in a few milliseconds is really constant, or whether there may not be a creep of potential which is too slow to register on the time scale used. Records showing the effect of pulses lasting 300 msec. were therefore made on a slow time base. The result was unequivocal, since the potential attained its maximum in a few milliseconds and then remained constant for the duration of the pulse.

Experiments with dead nerve fibres

Measurements of the extrapolar potential are liable to be complicated by errors and artifacts of various kinds (cf. Bogue & Rosenberg 1934). A number of control experiments were therefore made in order to ensure that the potential recorded in the extrapolar region was entirely due to accumulation of charge at the nerve membrane. In general, we found that the spread of potential in the extrapolar region was reduced progressively as the fibre lost its physiological activity, and that it finally fell to a low value when the fibre became inexcitable. A very striking demonstration of this general type of behaviour can be obtained by allowing the axon to come into contact with a solution of chloroform. Figure 6 illustrates an experiment of this kind. Records *b-g* show the spread of potential in the extrapolar region of a normal axon, and demonstrate the passive accumulation of charge at the surface membrane. The fibre was then dipped into sea water which had been shaken with chloroform. It was left in this solution for 1 or 2 min. and raised into oil. The result was extremely striking; for the potential change at the cathode was reduced to one-twentieth of its former value and was abolished at all other points. Records *a* and *A* are an index of the current through the axon, which was unchanged by the chloroform treatment. This experiment illustrates the delicate nature of the surface membrane and provides a convincing demonstration of the virtual absence of artifacts. The small potential which is recorded at the cathode in *B* may be attributed either to a residual membrane resistance or to the finite thickness of the nerve fibre. Close examination of the original records revealed a rapid spike which occurred at the beginning and end of the square wave, but was too faint for

reproduction. This persisted after chloroform treatment and must be regarded as an artifact caused by capacitive coupling between the polarizing and recording leads. The spike was ignored in analysing the records, but served a useful purpose in defining precisely the beginning and end of the applied current.

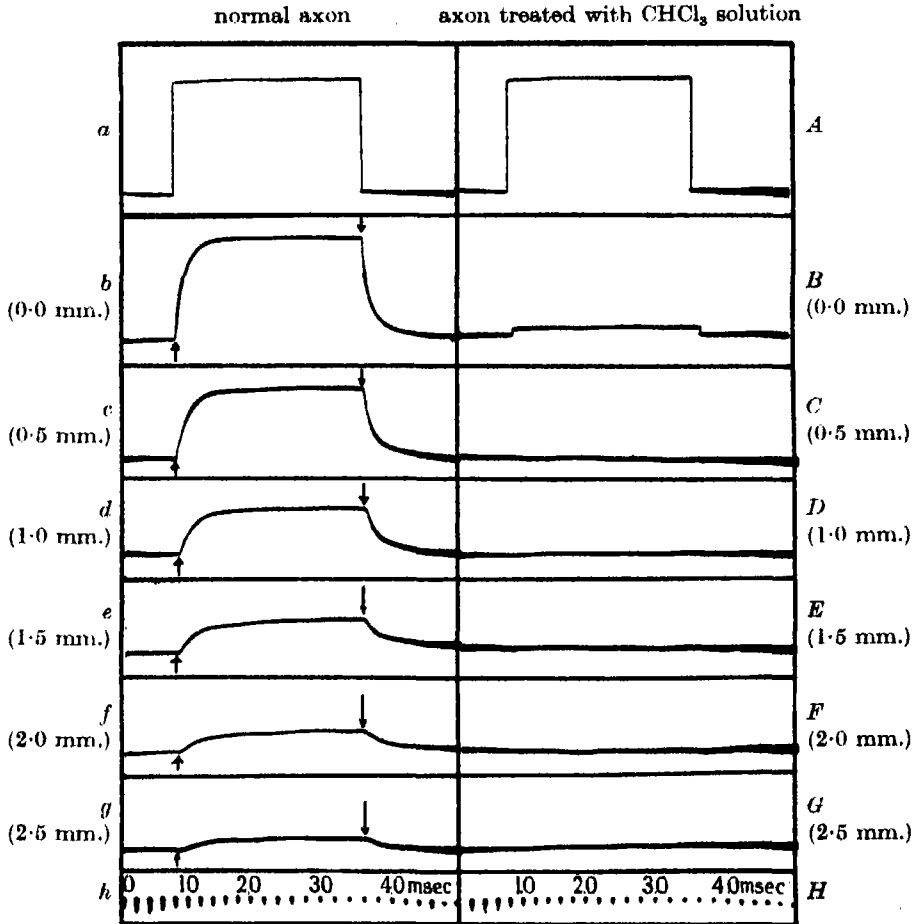


FIGURE 6. Effect of chloroform solution on spread of membrane potential in *Homarus* axon. *a*, *A*, current through normal and chloroform treated axon, measured as voltage across $61,700\Omega$ resistance in series with axon; *b*–*g*, potential recorded in extrapolar region of normal axon; the distance from the cathode is shown by the figures in brackets; *B*–*G*, potentials recorded in the same way after application of chloroform solution; *h*, *H*, 500 cye./sec. time calibration. The vertical arrows indicate the beginning and end of the square wave of current and were marked from a capacitive artifact which appeared on the original records. Records *a* to *c* have been retouched. The amplification was the same in all records and the amplitude of the wave in *b* was approximately 4.5 mV. Records taken from experiment 13.

The measurement of λ

Equation (6.2) shows that there should be an exponential relation between the steady potential in the extrapolar region and the distance from the cathode. Hence a straight line with slope $(\log_{10} e)/\lambda$ should result when the \log_{10} of the

potential is plotted against distance. This method was used in all the experiments and is illustrated by figure 7. In drawing a straight line through the experimental points, more weight was placed on observations near the cathode, since the percentage error increased as the recorded voltage decreased. Figure 8 proves that this procedure gave satisfactory results. Here the results of all the experiments are plotted on a linear scale: the ordinate giving the potential as a fraction of the potential at the cathode and the abscissa giving distance as a fraction of the space constant. If equation (6.2) were obeyed perfectly all the points should fall on an exponential curve which is drawn as a solid line. In practice there are deviations, but in no case are they at all serious. Hence this set of observations demonstrates the validity of the theory and of the method of measurement employed.

Table 2 shows that the average value for λ was 1.6 mm., but that its magnitude varied considerably in individual experiments. As will appear later the variations are primarily due to differences in the membrane resistance, and the scatter in the results reflects the variable nature of this quantity.

TABLE 2. ELECTRICAL CONSTANTS IN TEN AXONS FROM *HOMARUS VULGARIS*

experi- ment number	axon number	dia- meter μ	λ mm.	y $\Omega \times 10^3$	m $M\Omega \text{ cm.}^{-1}$	τ_m msec.	r_2/r_1	R_2 $\Omega \text{ cm.}$	R_4 $\Omega \text{ cm.}^2$	C_M $\mu\text{F cm.}^{-2}$	
1	1	65	1.80	78	0.72	1.6	0.82	43.6	1910	0.83	
2	2	80	1.07	49	0.80	1.8	0.87	75.2	927	1.94	
3	3	62	1.90	77	0.98	2.4	1.21	65.4	2784	0.87	
4	4	76	1.40	80	0.88	5.4	0.76	70.6	1655	3.24	
5	5	76	1.82	134	0.86	3.7	0.59	63.0	2955	1.25	
[{	6	73	2.95	103	0.90	4.0	1.3	83.6	7330	0.55	
	7	6	73	2.62	114	0.73	3.3	0.84	55.9	4590	0.71
	8	6	73	1.95	55	0.76	1.3	1.35	74.6	2720	0.46
	9	7	87	1.31	54	0.59	0.76	0.72	61.2	1150	0.66
{10	8	78	1.29	137	0.74	1.9	0.35	48.1	1590	1.23	
{11	8	78	0.81	112	0.71	0.91	0.26	43.2	706	1.29	
12	9	73	0.92	40	0.72	0.89	0.84	55.6	564	1.58	
13	10	80	1.15	55	0.66	2.5	0.69	56.6	905	2.73	
average value		75	1.61	81	0.83	2.3	0.81	60.5	2290	1.33	

Square brackets indicate that successive measurements were made on the same nerve fibre; curved brackets that they were made on the same stretch of the same fibre. Temperature: 15–20° C. Strength of current: 0.4–0.5 threshold. The values given for τ_m are the mean of four measurements.

The measurement of y

The constant y has the dimensions of a resistance and is given by the ratio of the steady voltage at the cathode to the applied current (see equation (6.1)). The method of measurement is clarified by referring to figure 6. Here b gives the voltage at the cathode and a the voltage across 61,700 Ω . Hence $y = 61,700 \times b/a \Omega$, where b/a is the ratio of the observed voltages. In this case y was 55,400 Ω , which was rather smaller than that usually obtained (see table 2).

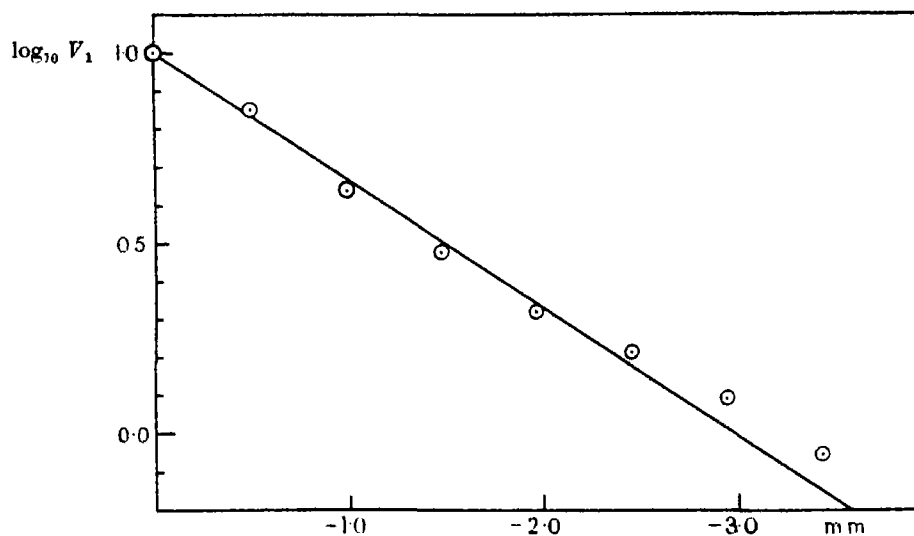


FIGURE 7. Equilibrium distribution of extrapolar potential. Ordinate: \log_{10} potential. Abscissa: distance of recording electrode from cathode in mm. The distance is shown as negative in order to conform to the convention used in the theoretical section.

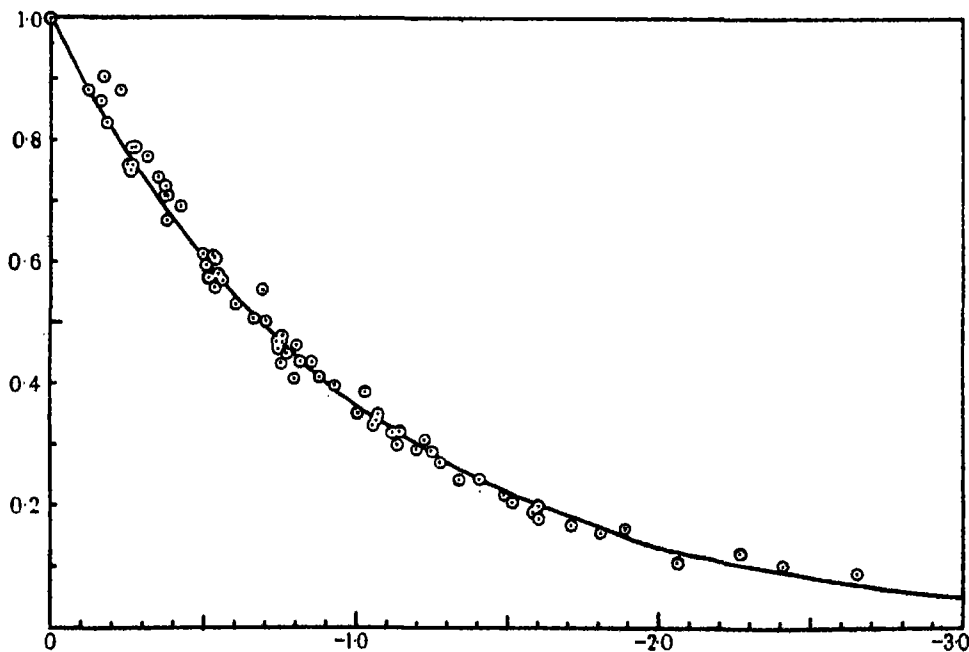


FIGURE 8. Equilibrium distribution of extrapolar potential in thirteen experiments. Ordinate: potential as a fraction of the potential at the cathode. Abscissa: distance as a fraction of the measured space constant λ . The solid line is drawn according to equation (6.2).

The measurement of m

m has been defined as the parallel resistance of core and external fluid. It was determined by measuring the voltage gradient midway between two distant electrodes and dividing the gradient by the current through the nerve. Typical records for determining m are given in figure 9. According to theory all these records should be perfectly rectangular, since the membrane impedance is not involved in the mid-interpolar region. The records actually show a slight creep, which can be explained in various ways. It might have been due to some capacitative property of the axoplasm or to irregularities in the diameter of the axis cylinder; or it could be attributed to the fact that the electrodes were not really an infinite distance apart as assumed in the theory. Whatever its explanation, the effect is not of present importance, since it makes little difference whether the maximum or the sudden rise is used for analysis. On the whole it seemed best to measure the sudden rise, since any effects introduced by the membrane were avoided by this procedure. The deflexion observed at any point could be expressed as a resistance by comparing it with the effect produced by the monitoring resistance. It was therefore possible to plot resistance against electrode separation as has been done in figure 10, which illustrates three typical experiments. The observed points fall very close to straight lines as they should according to theory. A direct measurement of m is given by the slope of the best straight line through the experimental points. The random nature of the errors involved seemed to justify a statistical treatment and m was therefore determined by the standard 'least square' formula.

The measurement of τ_m

The spatial and temporal distribution of the extrapolar potential are determined by the two constants λ and τ_m . λ has already been obtained so that τ_m can be determined by comparing experimental and theoretical curves. But first it must be established that the experimental records agree with the rather complicated equations of cable theory. Practice and theory are usually related by comparing experimental points with a theoretical curve. Here the situation is more complicated, since the experimental observations consist of a family of curves instead of a single set of points. In other words a three-dimensional surface has been found and must be compared with a theoretical surface. This imposes a much more drastic test on the theoretical equations, since only one parameter, τ_m , can be varied to make a number of curves coincide. In such a case it would be too much to hope for complete agreement at every point on the nerve. Nevertheless, agreement between theory and practice is reasonably good, as may be seen from figure 11. Here tracings of the voltage-time records at different distances are compared with the corresponding theoretical curves for those distances. Only a finite number of theoretical curves was computed and it was therefore impossible to use a theoretical curve which corresponded exactly with the experimental one. Thus C is the experimental curve for $x/\lambda = 0.38$ and d the theoretical curve for

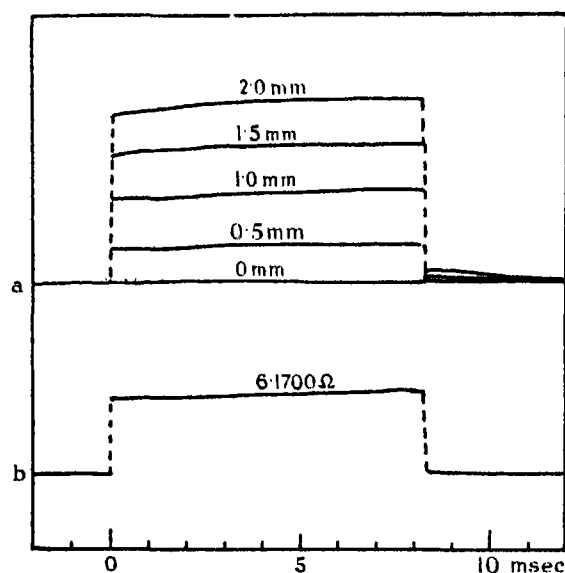


FIGURE 9. *a*, voltage gradient in mid-interpolar region. Records obtained with arrangement of figure 4*c* and with measuring electrodes separated by distances of 0–2.0 mm. *b*, voltage across $61,700\Omega$ using the same strength of current as that in *a*. Electrode arrangement as in figure 4*d*.

ohms $\times 10^3$

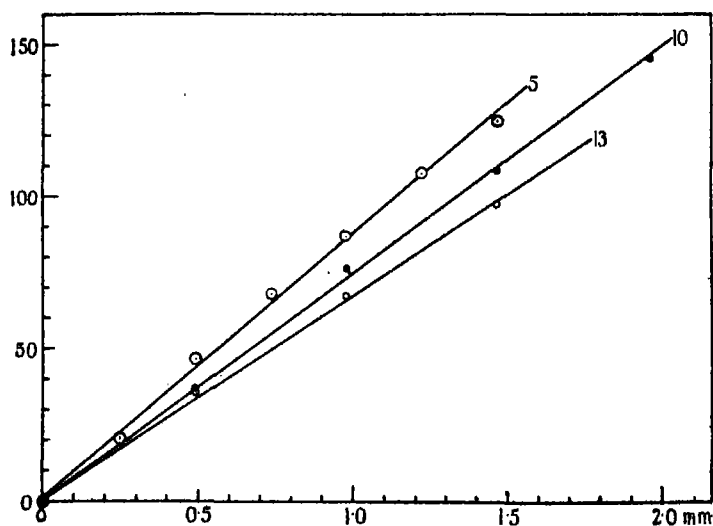


FIGURE 10. Resistance length relation in the mid-interpolar region. Ordinate: resistance, measured from records of the type shown in figure 9. Abscissa: distance between recording leads. The numbers on the straight lines refer to the experiments in table 2. The current was led into the nerve through electrodes about 16 mm. apart.

$x/\lambda = 0.4$. But the small differences introduced by this method of plotting do not materially alter the general picture of close agreement between theory and practice. Nor do they obscure the fact that there are certain real differences between the two sets of curves. Thus the record at the cathode rises more slowly than the corresponding theoretical curve, while the descending curves agree closely at the cathode but diverge at larger distances.

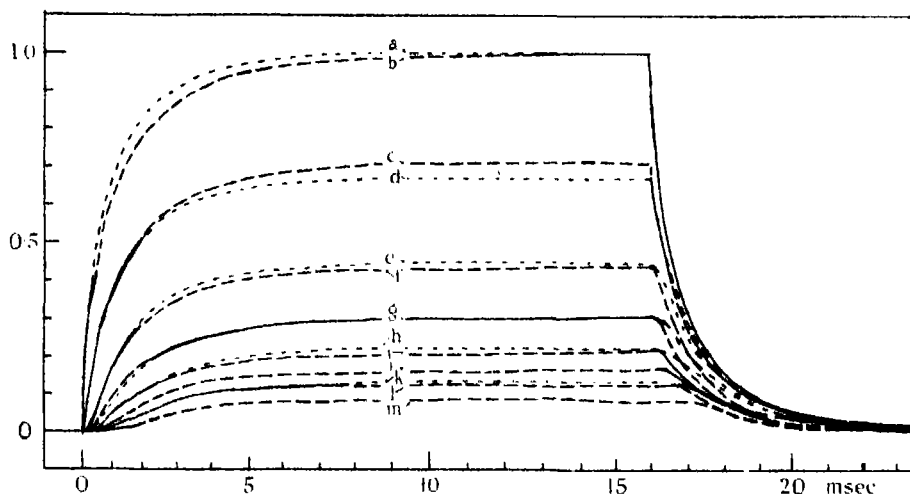


FIGURE 11. Experimental and theoretical curves showing rise and fall of extrapolar potential at different distances from cathode. Experiment 10 (table 2); $\lambda = 1.29$ mm. Abscissa: time in msec. Ordinate: potential expressed as a fraction of the equilibrium potential at the cathode.

- | | |
|--|--|
| a. Theoretical curve with $-x/\lambda = 0.0$ | h. Theoretical curve with $-x/\lambda = 1.5$ |
| b. Experimental curve with $-x/\lambda = 0.0$ | i. Experimental curve with $-x/\lambda = 1.52$ |
| c. Experimental curve with $-x/\lambda = 0.38$ | j. Experimental curve with $-x/\lambda = 1.89$ |
| d. Theoretical curve with $-x/\lambda = 0.4$ | k. Theoretical curve with $-x/\lambda = 2.0$ |
| e. Theoretical curve with $-x/\lambda = 0.8$ | l. Experimental curve with $-x/\lambda = 2.27$ |
| f. Experimental curve with $-x/\lambda = 0.76$ | m. Experimental curve with $-x/\lambda = 2.65$ |
| g. Experimental curve with $-x/\lambda = 1.14$ | |
| Theoretical curve with $-x/\lambda = 1.2$ | |

Theoretical curves drawn according to equations (6.0) and (6.4) with τ_m taken as 2.10 msec. Arrangement of electrodes as in figure 4a. Rectangular pulse with strength about 40 % threshold. The abscissa is not quite linear and the theoretical curves have been plotted according to the actual scale and not to a hypothetical linear scale; time calibrations derived from 500 cyc./sec. oscillator. A continuous line indicates that theoretical and experimental curves coincide.

The general coincidence between theory and experiment illustrated by figure 11 was only obtained because the theoretical curves were plotted with the correct time constant which in this case happened to be 2.10 msec. This value was obtained by a laborious process of trial and error which was too cumbersome for use in every experiment. It was therefore necessary to find a swifter method of computation. One possibility is to make use of the equations for total charge. This method was of little general use, but will be described briefly because it is of considerable

theoretical interest. Equations (5.2) and (5.3) show that the total charge obeys simple exponential laws. It follows immediately that the total extrapolar charge, which is proportional to $\int_{-\infty}^0 V_1 dx$, must also obey exponential charging laws. This quantity can be obtained by graphical integration of the potential in the extrapolar region and may then be plotted against time. The result of such an analysis is given in figure 12. Here the theoretical curve for the rise of a charge is drawn with a time constant of 2.02 msec. and for the fall with a time constant of 1.65 msec. The charging process obviously agrees closely with theory, but there is a definite deviation in the process of discharge. Further, the time constant for the charging process agrees with that found previously (2.10 msec.), whereas the discharge constant is appreciably smaller. The reason for these discrepancies is not clear, but they may arise from an apparently trivial circumstance. During the charging process the potential is relatively large and occupies a small area, whereas the converse situation holds during the period of discharge (see figures 2 *a, b*). This means that graphical integration is much less susceptible to cumulative errors in the former case than it is in the latter. The discharge curve may therefore be a less reliable index of the behaviour of the nerve than the corresponding charging curve. Whatever the explanation, this method will not be pursued further, since it proved too laborious for use in more than one experiment.

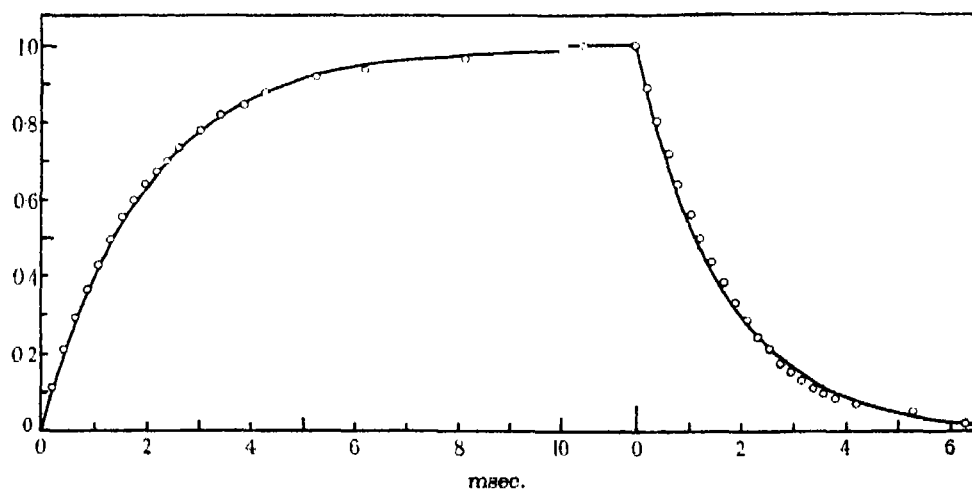


FIGURE 12. Time course of total membrane charge in extrapolar region. Abscissa: time in msec. Ordinate: $\int_{-\infty}^0 V_1 dx$ in arbitrary units. The circles are experimental points computed by graphical integration of photographic records from experiment 10. The solid line is a plot of equations (5.2) and (5.3) with $\tau_m = 2.02$ and 1.65 msec. for the rise and fall, respectively.

A simple method of measuring the membrane time constant is to ignore all observations except those at the cathode and find the time constant by comparing a single theoretical curve with the correct equation ((6.3) or (6.5)). This can be

done for both charging and discharging processes and has the advantage of simplicity. But it suffers from two serious disadvantages. In the first place it ignores a great deal of valuable information, and in the second it is liable to magnify the errors which arise from the finite width of the electrode. The general effect of electrode width is to lengthen the apparent time constant; for the effective cathode occurs on the interpolar side of the electrode and the effective recording point on the extrapolar side. With a cathode of width $\lambda/10$ the apparent time constant should be 10 % larger than the true time constant. But no worth-while correction can be made, since the exact current distribution at the electrode is unknown. Time constants measured by this method should therefore be regarded as only approximately correct.

Another method of measuring the time constant depends upon a remarkable property of equations (6.0) and (6.4). If the time to reach half-maximum is plotted against distance, a curve is obtained which is very nearly a straight line with a slope of $2\lambda/\tau_m$. An alternative statement of this result is that the half-value potential propagates at a constant velocity of $2\lambda/\tau_m$.^{*} Since λ is known, τ_m can be obtained by measuring the velocity of propagation from the experimental records.

Seven methods of measuring τ_m from the experimental data have now been described:

- (1) Trial and error to find best overall fit of experimental curves.
- (2) Rate of rise of total charge in extrapolar region.
- (3) Rate of fall of total charge in extrapolar region.
- (4) Rate of rise of potential at cathode.
- (5) Rate of fall of potential at cathode.
- (6) Propagation velocity of half-value potential following make of current.
- (7) Propagation velocity of half-value potential following break of current.

All seven methods were applied to one experiment, with the following results:

method	apparent time constant (msec.)	method	apparent time constant (msec.)
1	2.10	5	2.10
2	2.02	6	1.63
3	1.65	7	1.40
4	2.67	average	1.93

The last four methods were applied as a routine procedure to all the experiments, with results which are shown in table 3. The agreement between different methods is often poor and the variations seem to be entirely random in nature. But there is little doubt as to the order of magnitude of the time constant, and it is this that is of interest at the moment.

^{*} A footnote in Bogue & Rosenberg's (1934) paper suggests that this relation was known to Cremer.

TABLE 3. VALUES OF MEMBRANE TIME CONSTANT (τ_m) OBTAINED BY FOUR DIFFERENT METHODS

experiment number	τ_m in msec. determined by method				τ_m in msec. average
	4	5	6	7	
1	1.97	1.71	1.28	1.40	1.6
2	1.28	1.49	2.48	1.96	1.8
3	3.09	2.34	2.49	1.80	2.4
4	3.85	6.22	6.00	5.40	5.4
5	4.51	3.91	3.33	3.06	3.7
6	2.90	2.67	5.64	4.86	4.0
7	3.46	3.11	3.28	3.18	3.3
8	0.81	0.84	1.81	1.59	1.3
9	0.73	0.91	0.78	0.63	0.76
10	2.67	2.10	1.63	1.40	1.9
11	1.24	1.22	0.65	0.54	0.91
12	1.17	1.36	0.56	0.49	0.89
13	2.43	3.50	1.82	2.12	2.5
average	2.31	2.40	2.44	2.19	2.34

The relative magnitude of internal and external resistances

The ratio of the internal to external resistance per unit length (r_2/r_1) is important, because it allows us to estimate the absolute magnitude of potential changes at the nerve membrane. Equation (1.4) was derived without reference to the properties of the surface membrane, and it may therefore be applied to any region of nerve which does not form part of an external circuit. In general

(potential change at membrane)

$$= (\text{potential change recorded externally}) \times (1 + r_2/r_1).$$

r_2/r_1 was obtained from the experimental results by equation (8.3) and calculated values are given in table 2. Action potentials were measured in five of these experiments, and the absolute magnitude of the electrical change at the surface membrane could therefore be estimated. The average value for the membrane action potential was found to be 110 mV and the extremes 135 and 87 mV. This result is in good agreement with the direct measurements which have been made with a micro-electrode in squid axons (Curtis & Cole 1942; Hodgkin & Huxley 1939).

The measurement of r_2/r_1 was subject to a small systematic error. In the theory it was assumed that the electrode was infinitesimal in width, whereas it actually had an effective width of 100–150 μ . The measured value for r_2/r_1 would therefore exceed the true value by an amount which we estimate roughly at 10 %.

The axoplasm resistivity (R_2)

The resistivity of the axoplasm can be computed by equation (8.0):

$$R_2 = \pi a^2 m (1 + m\lambda/2y).$$

It would be unwise to expect great accuracy or consistency in the calculated value of R_2 , since four separate measurements enter into its determination, and the final

result is subject to the errors which arise from the assumption of infinitesimal electrode width. A rough estimate of the total error in R_2 is that it amounts to $\pm 30\%$. Table 2 shows that the average value of R_2 was 60.5Ω cm. and the limits 43.2 and 83.6Ω cm. The average value of the axoplasm resistivity was, therefore, about three times as great as that of the surrounding sea water. This result is similar to those obtained for other cells. Measurements with transverse electrodes gave an average value of four times sea water for the resistivity of squid axoplasm (Cole & Curtis 1938), and observations with axial electrodes an average of 1.4 times sea water for the same material (Cole & Hodgkin 1939). Red and white blood corpuscles have a resistivity of twice plasma, frog's sartorius muscle one of about three times Ringer and various echinoderm eggs a resistivity of four to eleven times sea water (for references see Cole & Cole (1936) and Bozler & Cole (1935)).

The membrane resistance

The resistance \times unit area of the surface membrane is determined by equation (8.1):

$$R_4 = 2\pi a\lambda^2 m(2 + m\lambda/2y + 2y/m\lambda).$$

Table 2 shows that the ratio r_2/r_1 which is equal to the factor $m\lambda/2y$ usually lies between $\frac{2}{3}$ and $\frac{3}{2}$. This means that a large error in $m\lambda/2y$ will have only a small effect on R_4 . Suppose, for example, that the true value of $m\lambda/2y$ is 1.0 and that it is measured as 1.5. In the first case the factor in brackets in (8.1) would be 4.0 and in the second 4.17; hence the error in R_4 would only be 4%. A similar line of argument shows that the measured value of R_4 will only be very slightly affected by the assumption of infinitesimal electrode width. The accuracy of the R_4 determination is, therefore, primarily controlled by the measurement of λ^2 , a and m . The errors in λ^2 are likely to be of the order of $\pm 30\%$, and almost certainly swamp the errors in a and m . A conservative estimate of the accuracy of the measurements in table 2 is that the values given for R_4 are correct to within 50%. The observed variation was much greater than this, and successive measurements on one axon showed that the membrane resistance declined progressively during the course of an experiment. Thus axons 6 and 8 had initial resistances of 7330 and 1590Ω cm.², while their final resistances were 2720 and 706Ω cm.². The variable properties of the surface membrane mean that an average or standard value cannot be given for its resistance. All that can be said is that axons with resistances varying from 600 to 7000Ω cm.² are capable of conducting nervous impulses in a normal manner. It is equally impossible to estimate the value of the membrane resistance in the living animal. The natural membrane resistance is not likely to be less than that found *in vitro*, but it may be much higher since Blinks's (1930) work on *Valonia* indicates that the surface resistance falls when cells are handled.

The values for R_4 given in table 2 are considerably larger than those recorded in the squid axon. Cole & Hodgkin (1939) reported values ranging from 400 to 1100Ω cm.² on the basis of resistance-length measurements with direct current, while Cole & Baker (1941a) obtained an upper limit of 200Ω cm.² from measure-

ments with a.c. and transverse electrodes. On the other hand, Cole & Curtis (1941) give an average value of only $23\Omega\text{ cm.}^2$ from measurements with an internal electrode and d.c. pulses. Finally, Cole & Baker (1941*b*) calculated a value of $350\Omega\text{ cm.}^2$ from the result of a.c. measurements with longitudinal electrodes and the assumption of a membrane capacity of $1.1\mu\text{F cm.}^2$. Cole (1941) appears to regard $300\Omega\text{ cm.}^2$ as a more or less average value. The low value of $23\Omega\text{ cm.}^2$ was attributed by Cole & Curtis (1941) to the poor physiological condition of impaled axons, but as they point out it may also have been due to the fact that two constants required in the analysis were assumed and not measured. In any case, there seems to be no doubt that the membrane resistance of 75μ lobster axons is several times larger than it is in 500μ squid axons. This difference may have some functional significance, since the rate of attaining ionic equilibrium tends to increase with surface-volume ratio, other things being equal. The membrane resistance would therefore need to decrease as the diameter increased if the cell economy demands a constant rate of approach to equilibrium.

The values of membrane resistance encountered in our work suggest that the permeability to ions must be rather low. Some idea of this may be gained by supposing that potassium ions alone can diffuse through the membrane and that permeability is studied by replacing the potassium in the external solution with a radioactive isotope. In this case it is fairly easy to show that approximately 30 min. would elapse before an 80μ fibre with a membrane resistance of $7000\Omega\text{ cm.}^2$ reached a state in which one-tenth of its internal potassium was replaced by the radioactive isotope. It would be interesting to see whether the rate of penetration of potassium is of this general order of magnitude.

Our values for the membrane resistance may be compared with those obtained by Dean, Curtis & Cole (1940) on artificial films containing lipoid and protein molecules. These films were of the right electrical thickness, since their capacity was about $1\mu\text{F cm.}^2$, but their electrical resistance was only $50\text{--}100\Omega\text{ cm.}^2$. It is too early to try to correlate this difference with chemical structure, but there is some hope that future work will show what sort of structure is needed to produce a membrane of high resistance.

The magnitude of the membrane capacity

The membrane capacity was determined by the relation

$$C_M = \tau_m/R_4.$$

Both τ_m and R_4 are subject to large errors, so that little confidence can be placed on the exact numerical values obtained for C_M . In fact, it is possible that the variation encountered in table 2 was entirely due to experimental error. But there can be little doubt that the membrane capacity was of the order of $0.5\text{--}2.0\mu\text{F cm.}^2$. A value of this kind has been obtained in a wide variety of living cells; well-known examples are red blood cells $0.95\mu\text{F cm.}^2$, yeast $0.60\mu\text{F cm.}^2$, echinoderm eggs

$0.87\text{--}3.1\mu\text{F cm.}^{-2}$, frog's sartorius muscle $c. 1\mu\text{F cm.}^{-2}$, squid nerve $1.1\mu\text{F cm.}^{-2}$, and *Nitella* $0.94\mu\text{F cm.}^{-2}$ (for references and qualifications see Cole 1940).

All these results depend on the use of a.c., transverse electrodes and a theory based on Maxwell's application of Laplace's equation to a suspension of spheres. Our observations were made with pulses of d.c., longitudinal electrodes and a theory based on Kelvin's equations for the submarine cable. So it is pleasing to find even a broad agreement between the two sets of results.

The implications of the membrane capacity of $1\mu\text{F cm.}^{-2}$ are too well known to be repeated. All that need be said is that the result suggests the presence either of a very thin membrane, or of one with a large dielectric constant. If the dielectric constant were 3, the membrane thickness would be 27\AA ; and if the thickness were 1μ the dielectric constant would be 1100.

Possible membrane inductance

Cole & Baker (1941b) have presented experimental evidence which suggests that an inductive element is present in the surface membrane of the squid axon. No sign of inductive behaviour could be observed in the majority of our experiments. But the two sets of observations do not conflict in spite of the apparent contradiction. Cole & Baker's axons had a membrane resistance of about $300\Omega\text{ cm.}^2$, ours an average of $2300\Omega\text{ cm.}^2$. The effect of an inductive element would have been profoundly influenced by the value of the membrane resistance, since Cole & Baker's work indicates that the two elements are in series. To take a specific example: assume that the membrane has the equivalent circuit suggested by Cole & Baker, that the capacity is $1\mu\text{F}$, the inductance 0.2 H and the resistance $300\Omega\text{ cm.}^2$. When a rectangular current is applied to this circuit, the voltage response is oscillatory and the first overshoot is 75 % greater than the final steady value. The response is entirely different if the resistance is increased to $2500\Omega\text{ cm.}^2$. In this case the wave form is no longer oscillatory, it does not overshoot the steady value, and it differs from a simple exponential solution by less than 0.2 %. The absence of inductive or oscillatory behaviour therefore agrees with Cole & Baker's hypothesis, although it clearly cannot be used in evidence one way or the other. But some of the axons studied had low membrane resistances and should have shown signs of inductive behaviour, if Cole & Baker's picture is correct. This, in fact, is what happened. Figure 13c gives the response of an axon with a resistance of $700\Omega\text{ cm.}^2$ and shows that there is an overshoot of 5 %. There is no equation with which to compare this record, but a theory for total charge can be developed by the method used in deriving (5.1). The resulting expressions allow the membrane inductance (L) to be calculated from the overshoot and predict that the response will only be oscillatory when $L > R_M^2 C_M / 4$. Experiments 9, 11 and 12 (table 2) showed a small overshoot and gave an average value of 0.3 H for the membrane inductance. No overshoot was observed in the remaining experiments, and this is to be expected since the factor $R_M^2 C_M / 4$ always exceeded 0.4. Our results are therefore consistent with the existence of an inductive element of about 0.2 H cm.^2 .

But there is an entirely different way of explaining the experimental facts and this must now be considered. In figure 13 *a* and *b* the current had been increased until it was of just threshold strength, which means that it was strong enough to produce propagated spikes on 50 % of occasions. The propagated response is shown by *a* and the critical local response by *b*. It is arguable that the local response is of the same general nature as the spike, and that the discontinuity in nerve arises because the response to a superthreshold shock is large enough to involve the whole fibre by local circuit action, whereas the subthreshold response cannot spread beyond the cathodic region. It is also arguable that the small overshoot produced by the weak current is of the same general nature as the larger overshoot produced by the threshold current. And the similarity of the two lower curves in figure 13 suggests rather strongly that a common process is involved. According to this train of reasoning the overshoot seen in figure 13*c* is to be regarded as a vestige of the normal action potential. In this case it cannot be considered as an inductive effect. For the process underlying the action potential must involve energy liberation by the nerve, whereas a pure inductive overshoot would not. The two theories are therefore quite distinct, although no attempt can be made to decide between them until there are precise concepts to replace the general notions of inductance and energy liberation.

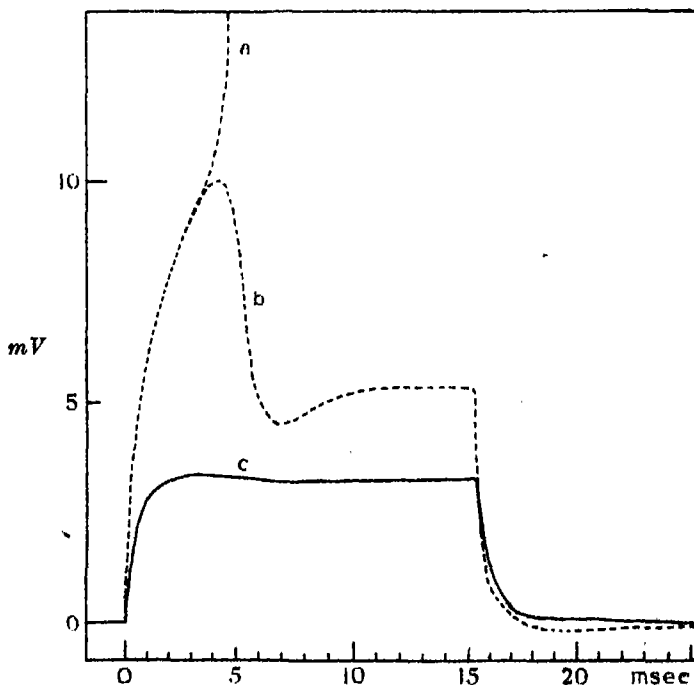


FIGURE 13. Potential recorded at cathode in axon with membrane resistance of $700\Omega\text{ cm.}^2$ (experiment 11). *a*, propagated response produced by current of strength 1.00; *b*, local response produced by current of strength 1.00; *c*, potential produced by current of strength 0.49. The absolute values given on the ordinate are approximate, but the scale is linear.

The idea of a membrane inductance is certainly useful, whatever its ultimate truth or falsehood. One application was found in the attempt to explain the difference between the action potential and the resting potential (Curtis & Cole 1942; Hodgkin & Huxley 1945). Another is illustrated by figure 14, which shows the effect of strong cathodic currents on a *Carcinus* axon. The records indicate that the wave form of the cathodic potential becomes increasingly oscillatory as

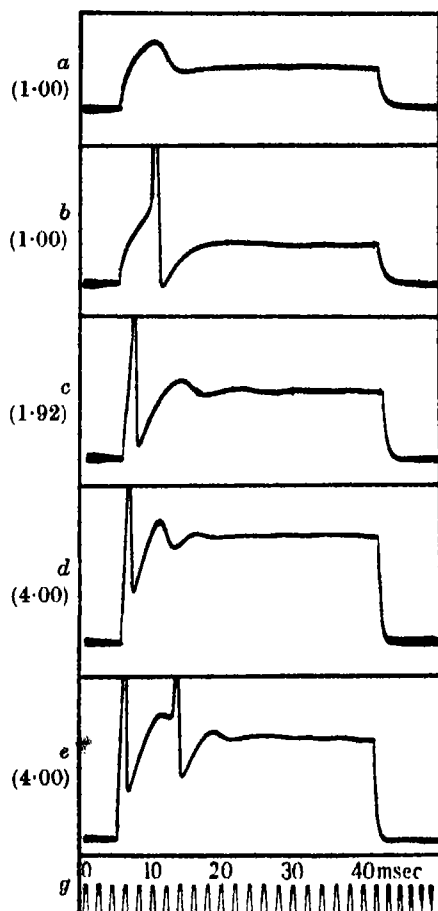


FIGURE 14. Effect of strong cathodic currents on *Carcinus* axon. Relative strength of current shown by bracketed figures. *g*, 500 cyc./sec. time calibration. Propagated spikes retouched.

Two points in figure 14 call for comment. After the oscillations have died out the membrane potential settles down to a steady value which is not proportional to the current but varies more slowly as the current is increased. This is an example of the membrane rectification described by Cole & Curtis (1941) in the squid axon.

In figure 14*e* a second spike arises from the second wave of potential, but a little after its crest. Hodgkin (1938) showed that a spike always started at a distance from the cathode when it arose later than the crest of the local response. Examination of the original records suggested that the same thing was occurring here, although no positive evidence for this conclusion was obtained.

the strength of current is increased. Similar results have been reported by Arvanitaki (1939) in *Sepia* and are to be expected from Cole & Baker's hypothesis. For the membrane resistance decreases progressively as the nerve is depolarized (Cole & Curtis 1941), and the response therefore becomes increasingly oscillatory as the current is raised. The frequency of the oscillations in figure 14 is consistent with an inductance of 0.3 H cm.^2 and a capacity of $2 \mu\text{F cm.}^{-2}$.

Observations on the local response

The most striking features of the local response were the inflexion and miniature spike which occurred when the duration of the rectangular wave was less than the utilization time. One example has already been described (figure 5), and a more general picture is given by figure 15 which shows the effect produced by threshold pulses of different duration. A large number of photographs were taken and with two exceptions only the responses which just succeed or just fail to propagate have been reproduced. The effect of a current longer than the utilization time is given by *c* and *C*. In this case the record shows first the passive charging of the nerve membrane and then a slow creep, which must be regarded as a local response, since it is absent from the anodic wave form (*a*). If the local activity succeeded in reaching a critical level it turned upwards and gave rise to a propagated action potential. When the critical level was not reached the response died out as a monophasic wave of low amplitude. The form of the propagating responses was not very different when the duration of the rectangular wave was less than the utilization time (*D* to *H*), but the responses which failed to propagate showed the characteristic inflexion and miniature spike (*d* to *h*). This type of response persisted as the duration of the current was reduced, but at very short times it changed to that characteristic of excitation by short shocks (*cf.* Hodgkin 1938). An example is given in *h* and *H*, but the details of the record cannot be appreciated on the slow time scale used. This set of records suggests that the condition for excitation by currents of different duration is that a critical potential must be reached; they also illustrate the reversibility of the process responsible for the action potential. One is accustomed to think that nothing can stop an impulse once the potential has begun to turn upwards into a spike. Our records indicate that the potential wave may fail to propagate, although it has shown the inflexion normally associated with a spike.

The records which have just been described were obtained from a *Carcinus* axon and may be regarded as typical of this preparation. *Homarus* fibres behaved in a similar manner, but the utilization time was considerably shorter and the rheobasic local response had a more conspicuous humped form. We obtained the impression that the long utilization time and flat local response were associated with a high membrane resistance, and that axons with a low resistance gave the short utilization time and humped local response characterized by figure 13.

In comparing our results with those obtained in whole nerve trunks it should be remembered that the amplitude of the subthreshold potentials was small compared

to the spike. Thus the propagated potential was ten times larger than the sub-threshold potential shown in figure 15. The effects we have described would therefore be difficult to observe in preparations giving spikes only $100\mu\text{V}$ in amplitude.

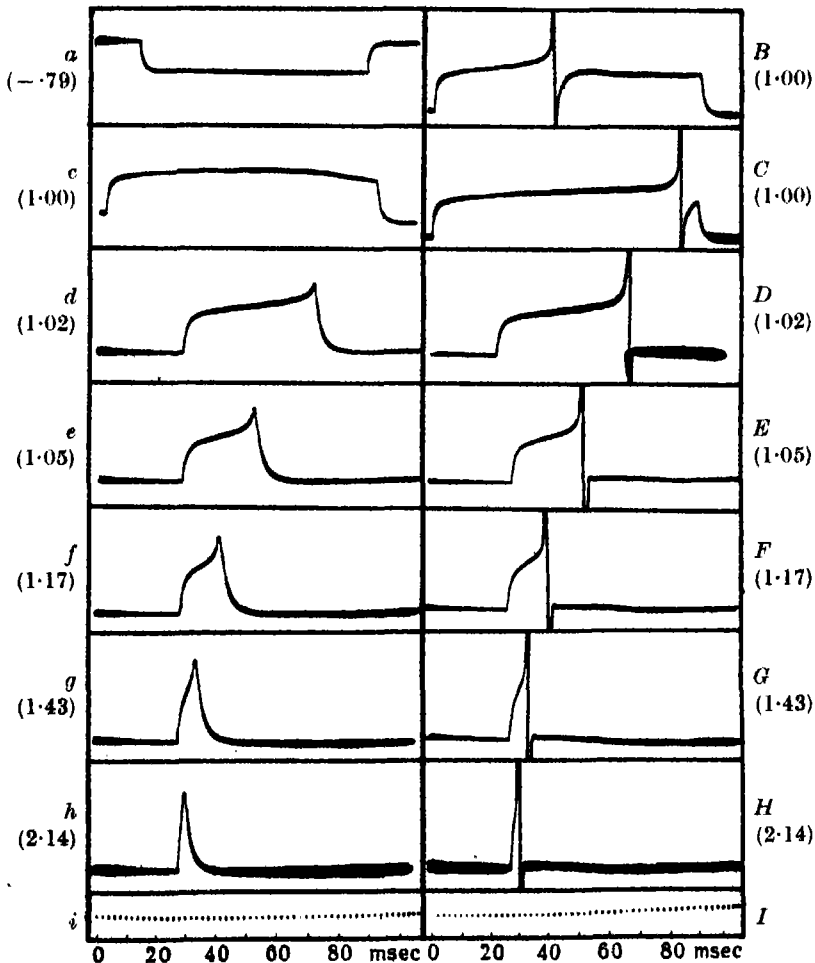


FIGURE 15. Effect of rectangular currents on *Carcinus* axon recorded at polarizing electrode. *a*, polarization produced by anodic current; *c-h*, local responses produced by threshold currents; *B-H*, propagated spikes (retouched) produced by threshold currents; *i*, *I*, 500 cyc./sec. time calibration. The strength of the current relative to the rheobase is indicated by the bracketed figures. The strength and duration of the current was identical in the pairs *c*, *C*, *d*, *D*, ..., *h*, *H*.

The change of membrane resistance during activity

A transient decrease of membrane resistance during activity has been proved by the well-known experiments of Cole & Curtis (1939). One result of this phenomenon is shown in figure 15*B*. Here the diphasic action potential arose before the end of the rectangular wave and lasted for about 2 msec. The membrane capacity should be discharged during the spike and must charge again when the resistance

returns to its normal value. The spike should therefore be followed by a charging process similar to that which occurred at the beginning of the rectangular wave. This effect is clearly shown by record *B* but is absent when the action potential arises at the end of the applied current (*D* to *H*). In this case the charge disappeared rapidly during the spike and did not reform, because the applied current was removed.

DISCUSSION

The implications of the resistance and capacity measurements have already been discussed. It remains to consider the bearing of our results on studies of electric excitation. The local responses observed in our experiments agree in a remarkable way with the instability described by one of us. Rushton (1932) studied the excitation process in medullated nerve by superimposing a short shock on a rectangular wave. A plot of excitability against time showed that the excitation process followed an inflected time course very similar to that observed in our records of local response. This is another example of the general similarity between the results of excitability studies on medullated nerves and the electrical records obtained in non-medullated nerve fibres. The phenomenon of latency and the excitability effects described by Rushton and by Katz (1937) all find an explanation in the electrical behaviour of isolated crustacean axons. The obvious conclusion is that similar electric effects exist in medullated axons, but that they are too small to be detected in studies of whole nerve trunks. This conclusion is not generally accepted and is likely to remain in dispute until satisfactory records can be obtained from an isolated medullated axon.

Hill (1936) and others have shown that many phenomena can be explained by supposing that the process of excitation is equivalent to the charging of a leaky condenser. This theory is useful in co-ordinating a wide range of observations, but extra assumptions have to be introduced to deal with the phenomena of accommodation, latency and the decay of excitability following a brief stimulus. Our results indicate that the processes underlying excitation are of great mathematical complexity. When the current is weak its spatial and temporal distribution is determined by the cumbersome equations of cable theory; when it is strong an immense complication is introduced by the non-linear effect of the local response. Hill's equations must therefore be regarded as largely empirical in nature. But there can be no doubt that certain facts seem to agree better with Hill's theory than with the cable equations. To take a specific example. It is universally agreed that the criterion for excitation by short shocks is that a fixed quantity of electricity must flow through the nerve. This follows at once from Hill's theory, but not from the equations of cable theory. For the condition which allows a short pulse to produce a constant potential at the cathode in a cable-like system is that a pulse of constant energy must flow through the electrodes. This difficulty and others of a similar kind can be resolved in the following way. The condition for excitation seems to be that the cathodic response must reach a potential at which it can propagate through the nerve by local circuit action. It is easy to suppose

that the criterion for propagation is related not to the membrane potential at the cathode but to the total membrane charge in the region of the electrode. In this case a constant quantity relation would be obtained and the behaviour of nerve would approximate to that of a leaky condenser in many respects. According to this view, Hill's 'local potential' is to be identified with the total charge in the electrode region and Hill's constant k with the membrane time constant. The true situation is obviously much more complicated, but this hypothesis provides a simple and convenient way of looking at the excitation process.

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The differential effect of synthetic growth substances and other compounds upon plant species

II. Seed germination and early growth responses to some arylcarbamic esters and related compounds

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The preparation and biological examination of a number of arylcarbamic esters (arylurethanes) are described. The experiments demonstrate the marked effect of *isopropyl phenylcarbamate* and some related compounds in very low concentrations upon the germination and seedling growth of cereals. The absence of effect of the same compounds in similar concentrations upon some dicotyledonous plants is noted.

In a previous publication (Templeman & Sexton 1944) it was demonstrated that certain concentrations of a number of synthetic growth substances prevented germination or seriously restricted seedling growth of some plant species and produced no effect on others. In addition to the effects described in the previous paper (Templeman & Sexton 1944), which were concerned largely with materials known to affect the extension growth of cells, the responses to substances known to interfere with nuclear division in plants have been investigated. Colchicine is perhaps the best known substance of this type and there is now a very wide literature dealing with it; chloral hydrate (Blakeslee 1937; Gavaudan & Gavaudan 1939 *a*; Shigenaga 1937), acenaphthene (Gavaudan, Gavaudan & Durand 1938 *a, b*; Kostoff 1938 *a, b*; Nebel 1938; Shmuck 1938; Schmuck & Gusseva 1939, 1940 *b*; Schmuck & Kostoff 1939) and a number of other materials have been shown by several authors (Favorsky 1939; Gavaudan & Gavaudan 1939 *b, c, d*; Gavaudan, Gavaudan & Durand 1939 *a, b, c, d*; Schmuck & Gusseva 1940 *a*; Simonet & Guinochet 1939) to arrest or interfere with mitosis. Lefevre (1939) has described the effects of ethyl phenylcarbamate ('phenylurethane') upon wheat seedlings. He considers the effects to resemble those described for colchicine—i.e. morphologically, bulbous hypertrophies of the coleoptile, the mesocotyl and the root tip with progressive slowing down or even final arrest of growth (manifestations greatly accentuated in seedlings treated before germination); and cytologically, blocking of mitoses in pseudo-metaphases to lead more or less gradually to an irregular formation of monstrous restored nuclei which are deformed, plurilobed or multiple.

During a series of preliminary experiments with cereal seeds and seedlings ethyl phenylcarbamate arrested growth at lower concentrations than colchicine, chloral hydrate or acenaphthene. Therefore attention was concentrated on arylcarbamic esters as a type, and this paper records observations made with arylcarbamic esters ('arylurethanes') of the general formula $\text{Aryl NH} \cdot \text{COO alkyl}$, together with certain related types including substances where the oxygen atoms are replaced by sulphur.

I. PRELIMINARY EXPERIMENTS

The effect of ethyl phenylcarbamate upon the germination and seedling growth of cereals was first noticed in an experiment carried out in December 1940. This compound was applied at a rate corresponding to 25 lb./acre to boxes in which spring oats and yellow charlock (*Brassica Sinapis* Vis.) had been sown in soil. The application was made immediately following seed sowing, and was sprayed in aqueous solution at a rate equivalent to 100 gal. of solution per acre. The charlock germinated and grew normally as in the controls which were untreated, but the oats showed an interesting effect. The seeds germinated and the first leaf appeared through the soil; then, however, growth was arrested—the first leaf ceased to grow in length, but it and its leaf base became very much thickened and no further leaves appeared. Root growth apparently ceased, and after remaining in this static condition for some time the seedlings eventually withered and died.

In a second experiment ethyl phenylcarbamate was applied again as an aqueous spray, but this time at rates equivalent to 1, 5, 10 and 25 lb./acre. Here the charlock was unaffected except that its germination was reduced by 25 lb./acre, but the effect on the oats was obtained with all the rates of application used. Once the growth of the oats was arrested it was never resumed and the seedlings succumbed. The marked effect of the lowest rate of application (10 mg./sq.ft.) indicated this substance to have marked physiological activity.

Later experiments showed that other cereals—barley, wheat, rye—were similarly affected as was perennial ryegrass, but that mangolds, sugar beet and potatoes were unaffected at the concentrations used. If the cereal crop was allowed to become established before the ethyl phenylcarbamate was applied to it, the damaging effect was less in amount but was still appreciable, and once the growth of even an established plant was completely arrested it did not subsequently renew growth.

The nature of this effect upon cereal seedlings as described also by Lefevre (1939) was very similar to that which is brought about by colchicine, by chloral hydrate and by acenaphthene. The relative activity of ethyl phenylcarbamate and these substances was therefore investigated. For instance colchicine at 25 lb./acre had no effect whatsoever on oats but did result in short, thick, stunted charlock plants. In several experiments in soil, colchicine was without effect on cereal seedlings and acenaphthene behaved similarly. Chloral hydrate diminished germination and stunted seedling growth of cereals, but larger amounts were required than of ethyl phenylcarbamate to bring about similar effects. Under laboratory conditions where oat seed was placed upon filter paper, maintained moist with the appropriate solutions, germination was retarded by 10–50 p.p.m. of the ethyl phenylcarbamate and completely inhibited by higher ones, whilst there was no shoot growth even at 10 p.p.m. For chloral hydrate 50 p.p.m. retarded shoot growth and 200 p.p.m. practically inhibited it.

From these preliminary results it was evident that ethyl phenylcarbamate was an extremely active compound toward cereal seeds and seedlings, and this led to the further chemical and biological investigation mentioned above.

II. PREPARATION OF COMPOUNDS

(a) *Arylcarbamic esters ('arylurethanes')*

Two general preparative methods have been employed: (i) interaction of the primary aromatic amine with the appropriate chloroformic ester, and (ii) interaction of phenylisocyanate with the appropriate alcohol. The compounds were purified either by crystallization or, in the case of liquids, by distillation.

The following substances have already been described in the literature, and their identity was confirmed by their physical characteristics:

methyl phenylcarbamate	ethyl phenylcarbamate- <i>p</i> -sulphonic acid
ethyl phenylcarbamate	ethyl phenylcarbamate- <i>m</i> -sulphonic acid
<i>n</i> -propyl phenylcarbamate	methyl <i>p</i> -nitrophenylcarbamate
<i>isopropyl</i> phenylcarbamate	ethyl <i>p</i> -nitrophenylcarbamate
<i>n</i> -butyl phenylcarbamate	ethyl <i>p</i> -methoxyphenylcarbamate
<i>isobutyl</i> phenylcarbamate	ethyl <i>o</i> -methoxyphenylcarbamate
allyl phenylcarbamate	methyl <i>o</i> -carboxyphenylcarbamate
cyclohexyl phenylcarbamate	ethyl <i>o</i> -carboxyphenylcarbamate
ethylene- <i>bis</i> -phenylcarbamate	ethyl <i>m</i> -carboxyphenylcarbamate
methyl α -naphthylcarbamate	ethyl <i>o</i> -chlorophenylcarbamate

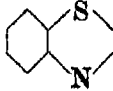
The properties of *isopropyl* phenylcarbamate call for comment. Its melting-point is recorded in the literature as 90 (Gumpert 1885) and 42–3° C (Spica 1887). We found a value of 89° C for material prepared by both methods and purified by repeated crystallization.

The following substances, not already recorded in the literature, were made by the same general methods. Their physical characteristics are given in the table below:

substance	crystallized from	m.p. (° C)
methyl <i>p</i> -chlorophenylcarbamate	methyl alcohol	65
methyl <i>p</i> -methoxyphenylcarbamate	alcohol	90
methyl <i>o</i> -methoxyphenylcarbamate	ether	29–30
methyl <i>m</i> -carboxyphenylcarbamate	methyl alcohol	221
methyl <i>p</i> -carboxyphenylcarbamate	benzene alcohol	197
ethyl <i>p</i> -carboxyphenylcarbamate	alcohol	201
<i>isopropyl</i> <i>o</i> -phenylcarbamate	alcohol	181
<i>isopropyl</i> <i>m</i> -phenylcarbamate	alcohol	205
<i>isopropyl</i> <i>p</i> -phenylcarbamate	benzene	198

(b) *Thiocarbamates*

Methyl and ethyl phenylthiocarbamates, $C_6H_5NH.CSOR$, were prepared as described in the literature from phenylisothiocyanate and the respective alcohols

R.OH; methyl benzthiazylthiocarbamate,  $C.NHCSOCH_3$, from thio-

cyanobenzthiazole and methyl alcohol (Davies & Sexton 1944); and methyl dimethyldithiocarbamate by reacting methyl sulphate with sodium dimethyldithiocarbamate.

(c) *Miscellaneous compounds*

Some of the compounds were already available and were examined for the reasons indicated below. Aniline and naphthylamine were tested to see whether the effects observed were mainly a function of the arylamino radicle or whether they were due to the molecule as a whole. Urethane itself, $\text{NH}_2\cdot\text{COOC}_2\text{H}_5$, and 'methyleurethane', $\text{NH}_2\cdot\text{COOCH}_3$, were tested for the same reason. Formanilide was tested because of its formal relation to phenylcarbamic acid and the arylsulphamic acids (made from the arylamines and chlorosulphonic acid), because of the analogy with the arylcarbamic acids. N:N-Di(carboethoxyaniline), $\text{C}_6\text{H}_5\text{N}(\text{COOC}_2\text{H}_5)_2$, was prepared in order to see whether the introduction of a second $-\text{COOC}_2\text{H}_5$ group had any effect on the biological activity. The two well-known hypnotics 'Sulphonal' and 'Trional' were tested to see how far the effects on seed germination were parallel to hypnotic activity.

III. *Biological experiments*

The compounds whose preparation was described in the previous section were examined for their effect upon oats in two ways. In the laboratory, oats have been germinated on filter paper kept moist with an aqueous solution of the test substance of concentrations up to 200 p.p.m. In boxes of soil, the test substance has been applied in sand immediately following the sowing of the seed at rates equivalent to 1-25 lb./acre. As a result of these tests a number of compounds have shown the property of preventing the germination of cereal seeds or of arresting seedling growth and causing thickening and malformation of the coleoptile and first leaf together with restriction of root growth when used in very low concentrations or amounts.

The compounds which have been discovered to be active in this way so far are listed below with some indications of their relative activities:

(1) isopropyl phenylcarbamate	the most active
(2) ethyl phenylcarbamate	about one-third as active as (1)
(3) N:N-Di(carboethoxyaniline)	
(4) allyl phenylcarbamate	slightly less active than (2) and (3)
(5) methyl phenylcarbamate	less active than (2) and (3)
(6) ethyl- <i>o</i> -chlorophenylcarbamate	
(7) <i>n</i> -propyl phenylcarbamate	
(8) chloral hydrate	less active than (5), (6) and (7)
(9) ethyl phenylthiocarbamate	
(10) methyl phenylthiocarbamate	slightly active
ethyl- <i>o</i> -methoxyphenylcarbamate	
(11) <i>n</i> -butyl phenylcarbamate	very slightly active

All the other compounds mentioned above were found to be inactive under the conditions of the experiments.

The isopropyl phenylcarbamate was thus proved to be an outstandingly active material. Under the somewhat artificial conditions of the greenhouse a rate of application equivalent to 1 lb./acre was sufficient completely to inhibit the growth of cereal seeds or seedlings. The following table gives the results of a laboratory experiment in which oat seeds were subjected by immersion to different concentrations of isopropyl phenylcarbamate in aqueous solution for different periods, rinsed and then put to germinate on filter paper maintained moist with distilled water. The times of treatment by each concentration to bring about the stated effects are given.

degree of effect	concentration in p.p.m.				
	1	5	10	20	50
reduction in root and shoot growth	2 days	16 hr.	< 16 hr.	1 hr.	< 1 hr.
root growth inhibited; shoot growth seriously reduced	3 days	1 day	16 hr.	2 hr.	< 1 hr.
complete inhibition of growth	not reached	2 days	1 day	4 hr.	4 hr.

Whilst the results quoted clearly indicate the effects on the cereal species one of the objects of this research was to determine whether ethyl phenylcarbamate and related compounds had different effects upon different plant species. Actual experiments showed that concentrations of ethyl phenylcarbamate and isopropyl phenylcarbamate which have very distinct effects on cereals are without effect on sugar beet, potatoes, mangolds, flax, rape and yellow charlock.

DISCUSSION

It is not proposed to enter here into a discussion of either the nature of the biological effects or of the variation in activity of the compounds with changes in chemical structure. Many more experiments have already been carried out and are continuing which will extend these preliminary observations. A previous communication (Templeman & Sexton 1946) detailed some synthetic substances which depressed the germination and growth of a number of dicotyledonous species at concentrations which had relatively little effect on grasses and cereals. The work described in this paper, however, with a different type of compound has given the reverse effect—the cereals being most readily destroyed with the dicotyledonous plants more resistant. The nature of the effect and the reasons why graminaceous plants are most susceptible to it are not yet clear, but since colchicine in higher concentrations does produce similar morphological changes in cereal seedlings, it is reasonable to anticipate that Lefevre's observations of disorganized mitosis will be confirmed for ethyl phenylcarbamate and that the more active isopropyl phenylcarbamate will be found to act in a similar way. The abnormal cereal seedlings reported by a number of workers (Brett & Dillon Weston 1941; Dillon Weston & Booer 1935; Dillon Weston & Brett 1940; Kostoff 1939, 1941; Nole 1938; Schwarz 1940) as the result of overdoses of organic mercury seed dressings appear to be similar to those which have been obtained in the experiments described in this

paper. It remains to be seen whether all the compounds which give the morphological effect are also active in inducing polyploidy.

Two types of series of compounds have thus emerged with very different effects upon different species brought about by very low concentrations. It is proposed to investigate further these observations both cytologically and physiologically for a range of plant species since they may serve as useful additional approaches to the more fundamental aspects of plant growth.

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The threshold of audition for short periods of stimulation

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It is shown that, in common with that for other sensory stimuli, the threshold of audition rises as the time of presentation of the stimulus is decreased. Investigation of the law relating change of threshold to period of stimulation suggests that it has a form similar to that found in the case of other sensory mechanisms.

INTRODUCTION

The threshold value of a sensory stimulus depends upon the period of time for which the stimulus is presented, only approaching a steady minimum value if that period is sufficiently great. This fact, and the complementary dependence of the intensity of a sensation upon the time during which the stimulus is applied, has long been known. In the case of vision, the classic experiments of Broca & Sulzer (1902) showed that the brightness of a small source of light increases considerably with the time of its exposure when that period is very short, reaches a maximum as the time of exposure is further increased, and thereafter falls off asymptotically towards a steady value for long-continued stimulation. This course of events runs parallel to the variations in the frequency of the pulses of action current in sensory nerve fibres; Adrian & Matthews (1927) have noted the similarity for the conger eye. The frequency of discharge plotted against the time after the beginning of steady stimulation shows the same rapid rise to a maximum and the same asymptotic fall to constancy. A similar phenomenon appears in the data given by Hallowell Davis (1935) for the auditory action currents of the cochlear nerve.

The rise of threshold as the time of stimulation is reduced is readily observed in the case of hearing. If an intermittent note is presented to the ear for, say, $\frac{1}{2}$ sec., and decreased in intensity until it is just inaudible, it will be clearly heard when it is made continuous. The change is of the order of 3 or 4 db. The present paper describes measurements made to show the existence of the phenomenon, and gives an account of attempts to find the law governing this change of threshold with period of stimulation for pure tones of various frequencies.

APPARATUS

The pure tones were produced by a heterodyne oscillator followed by an amplifier of maximum output about 500 mW incorporating two independent controls of intensity. The sound was presented by a telephone earpiece forming part of the output network of the amplifier and connected to it for the required time by the switch described below. The output circuit is shown in figure 1.

The observer sat in a 'sound-proof' chamber, with a sponge-rubber pad between his ear and the earpiece to ensure complete comfort in protracted periods of

listening; a small aperture in the pad allowed sound to reach the ear. A switch enabled him to cut off the sound when necessary. A microphone enabled him to communicate with an assistant outside who controlled the apparatus and who could also signal back to the observer when he was about to begin a set of stimuli.

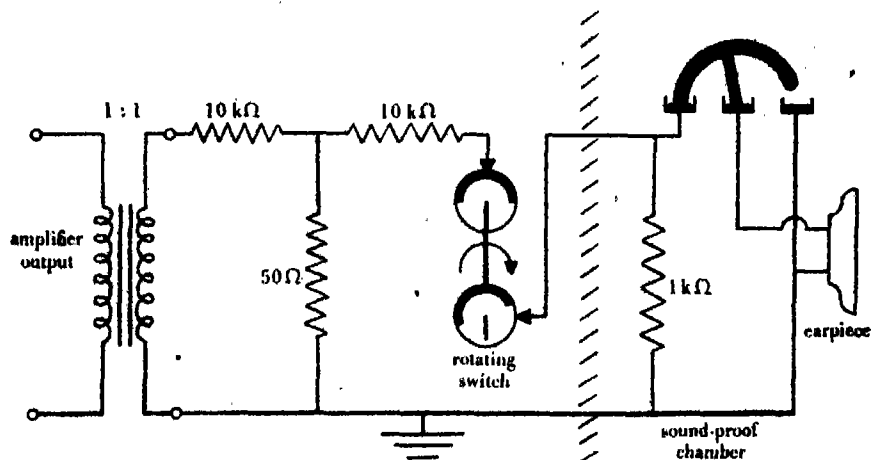


FIGURE 1. Amplifier output circuit.

The switch for presenting the note for a definite period consisted of two disks of insulating material, 3 in. in diameter; to each of these were bolted semicircular pieces of sheet metal to which had been soldered metal strips, 0.5 in. wide, to form a semicircular flange 4 in. in diameter. The metal was cut away at the centre of the disks, and bush wheels were bolted to the latter so that they could be mounted on a common axle. The flanges were balanced by lead weights bolted to the disks. Each flange was faced with thin copper foil, and two strips of flexible spring about 3 in. long were fixed by terminal screws to an insulating base to act as brushes, each making contact with the copper foil of one flange during half the period of rotation of the corresponding disk. The flanges were electrically connected, so that the time for which a conducting path existed between the terminals could be varied by adjusting the relative angular positions of the disks on their common axle. The maximum time was thus about half the period of rotation of the disks. These were driven (through reduction gearing) by a synchronous motor operating from the 50 cyc./sec. a.c. mains, and revolved once in every 1.42 sec. Seven different time-settings were provided by fitting a peg in one disk into one of seven holes in the other; changes of duration of the note heard could thus be readily made and repeated. These seven durations were determined by a kymograph and a time-marker recording fiftieths of a second. The traces could thus be measured to 0.005 sec. with ease. Five groups of three consecutive switchings were timed for each position of the peg; all were mutually consistent. The seven durations used in the experiments were usually 0.063, 0.117, 0.177, 0.241, 0.427, 0.611 and 0.789 sec. In earlier experiments the surfaces of the flanges were lightly lubricated

with vaseline; later, amalgamation of the surfaces was found to give better results. The switch timings were remeasured after amalgamation. Either method gave freedom from stray clicks in the earpiece at make or break, but the latter perhaps maintained this for longer runs without attention.

PROCEDURE

The determination of the settings of the intensity controls at the monaural threshold was carried out as in previous work (Hughes 1938). A series of such settings was made for any one frequency, starting with the shortest presentation of the note, and passing progressively to the longest, finally returning to the shortest as a control upon the set of determinations. If the initial and final measures for this shortest presentation differed by more than 2 db. the whole set was rejected. This criterion was adopted on the basis of an uncertainty of 0.5 db. found previously (Hughes 1938) for individual settings for a given time of stimulus. During the whole series of readings the observer remained undisturbed in the chamber and did not change the position of the telephone upon his ear, thus avoiding possible variations from this source. Determinations were made by two observers (J. W. H. and J. H. S.) at frequencies of 250, 500, 1000, 2000 and 4000 cyc./sec. At each of these frequencies ten series of readings were made by the former, and five by the latter. The mean of the two settings for the shortest duration was taken as the reference level, and the readings for the other (longer) durations were expressed as decibels below this level.

RESULTS

The average value of threshold, in decibels below the reference level, was calculated for each duration of each note separately for each observer. These averages, when plotted against the duration of the note, were found to lie on a smooth curve. When the averages in decibels were converted to energy ratios, i.e. the ratios of the threshold intensity at any duration to that at the shortest duration, and these ratios were plotted against the reciprocals of the times of presentation, the curves became straight lines. This is shown in figures 2 *a* and *b*.

Thus, if the threshold intensity for any duration t sec. is I , and that for the shortest is I_0 , it appears that the relation between these quantities is of the form

$$I/I_0 = b + a/t,$$

where a and b are constants for the given frequency.

If it be assumed that the law holds when the presentation of the note is continuous, i.e. when $t = \infty$, and that the threshold intensity is then I_∞ , then $b = I_\infty/I_0$, so that

$$I = I_\infty(1 + a/bt) = I_\infty(1 + \tau/t), \quad (1)$$

where τ is a constant of the dimensions of time.

Save that I and I_∞ are energies per sec. instead of current strengths, this is of the same form as the chronaxie equation of Lapicque (1926), and τ may be regarded

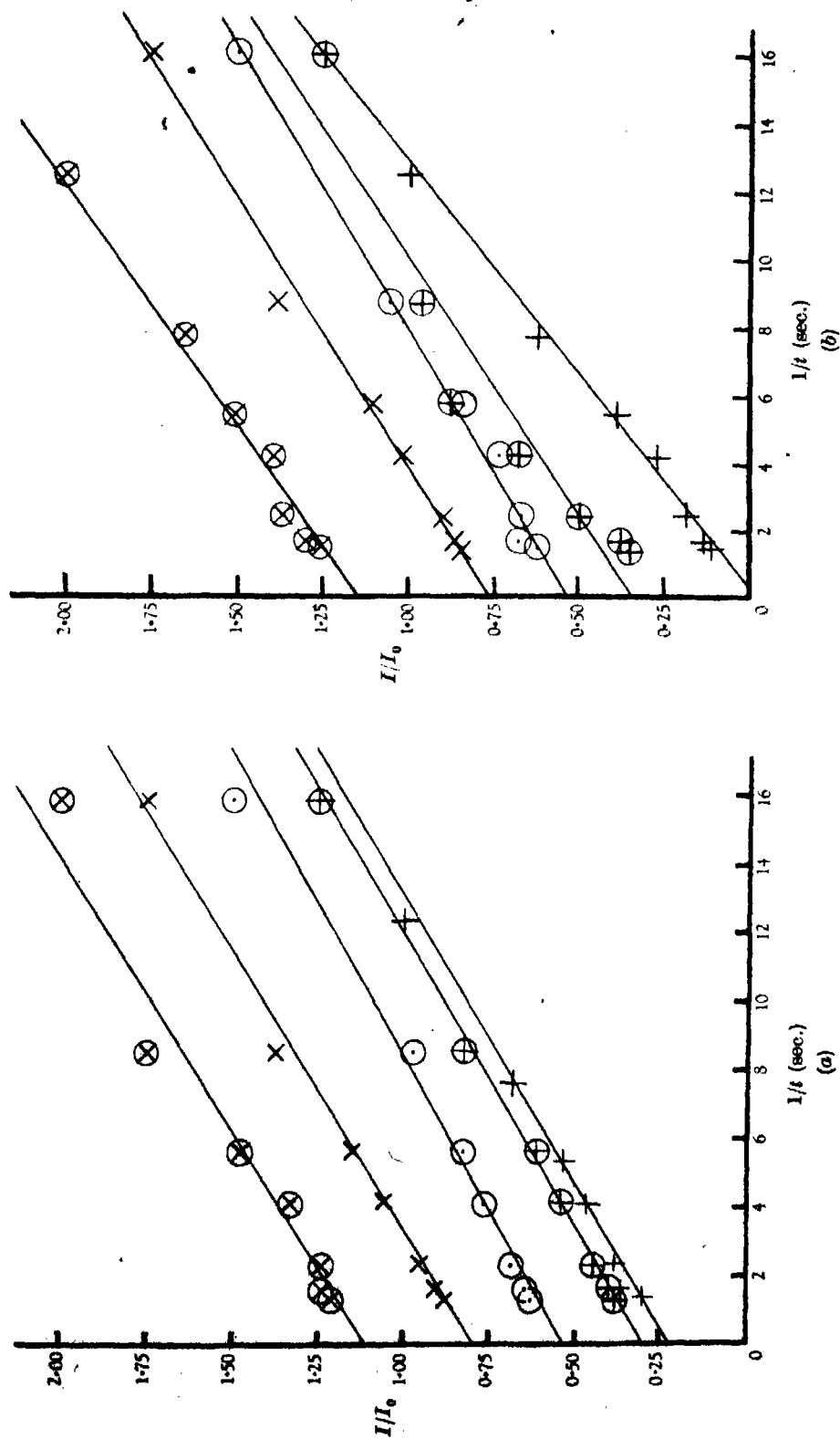


FIGURE 2. Energy ratio plotted against $1/t$ for different frequencies and observers. (a) J. W. H. (b) J. H. S. \otimes 500 cyc./sec. \odot 1000 cyc./sec. \times 2000 cyc./sec. \oplus 4000 cyc./sec. Vertical scale for 250 cyc./sec.; ordinates for successive curves increased by 0.25 for clarity.

as a pseudo-chronaxie for the sensory mechanism concerned, i.e. the time required for stimulation by an intensity twice that of the basic threshold intensity for a continuous stimulation.

DISCUSSION

In the case of the observations for each single frequency, all the data satisfy equation (1) within the limits of experimental error. It is more difficult to draw definite conclusions as to the variations of a , b or τ with change of frequency. In the first place these different frequencies were necessarily studied at intervals of several hours, usually on different days, and the individual threshold is certainly subject to appreciable, though not very large, changes under such circumstances. Secondly, the linear form suggested may not hold for long periods of presentation; this point was not tested, as the experiments were completed before any plotting other than that of decibels had been attempted, and the linear relation had not yet been noticed. (The outbreak of war prevented further work.) Even more important is the fact that though the relative level in decibels during a series was definite, the absolute level with respect to a fixed physical standard was much less sure. Hence, though the slope of the straight lines is relatively accurate in all the graphs, the intercepts with the axes are less determinate. In other words, the constant a of equation (1) can be found with fair precision, but the value of b , and therefore of τ , is more uncertain.

These conclusions would appear to be borne out by the results. The readings made with J. W. H. as observer were carried out with the greatest care and with every precaution of leisurely determination. Those made with J. H. S. were of the nature of a general confirmation, were fewer in number (five sets at each frequency instead of ten) and were made much more rapidly. Now the slopes found for J. H. S. are nearly equal to those for J. W. H.'s results, but his intercepts are much more irregular; in fact, some suggest that the line passes near the origin or even intercepts the intensity axis at small negative values. But if attention is confined to the data found for J. W. H., it appears that the intercepts are more consistent, though there is a distinct rise in their value at the extremes of the frequency range investigated. Without further experimental evidence, however, it would be unwise to make any remarks as to the possible significance of this.

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